REMARKS

Applicants thank the Examiner for his review of the instant application. Claims 27 and 32-35 are presented for examination. Applicants respond below to the rejections set forth in the final Office Action dated July 19, 2004 and maintained by the Examiner in the Advisory Action mailed November 8, 2004.

Rejection under 35 U.S.C. § 101 – Utility

The Examiner maintains the rejection of Claims 27, and 32-35 under 35 U.S.C. § 101 as lacking patentable utility. The Examiner asserts three reasons why the previously submitted Polakis declaration is unpersuasive. First, the Examiner maintains that the Polakis declaration addresses the correlation between increased mRNA levels and increased protein levels, and that the specification allegedly does not contain information regarding increased mRNA levels of PRO1800. Next, the Examiner asserts that the Polakis declaration does not provide data regarding the conclusions therein. Finally, the Examiner alleges that there is no evidentiary support to Dr. Polakis' testimony that it remains a central dogma in molecular biology that increased mRNA levels are predictive of corresponding increased levels of the encoded polypeptide. The Examiner also dismisses the previously submitted Pollack, Orntoft and Hyman references as evidentiary support for utility, arguing that since the references post-date the instant application, Applicants cannot rely upon them to establish the state of the art at the time the invention was made. Finally, the Examiner dismisses the Hanna reference as irrelevant to the instant application, as it deals with a known tumor-associated gene.

Applicants submit that the evidence in the record, in addition to evidence submitted herewith illustrating that at the time the invention was made it was well-known in the art that gene amplification correlates with increased levels of the encoded gene product, confirm that Applicants have established credible, substantial and specific utility for PRO1800 polypeptides.

Utility – Evidentiary Standard

An Applicant's assertion of utility creates a presumption of utility that will be sufficient to satisfy the utility requirement of 35 U.S.C. § 101, "unless there is a reason for one skilled in the art to question the objective truth of the statement of utility or its scope." *In re Langer*, 503 F.2d 1380, 1391, 183 USPQ 288, 297 (CCPA 1974). See, also *In re Jolles*, 628 F.2d 1322, 206 USPQ

885 (CCPA 1980); *In re Irons*, 340 F.2d 974, 144 USPQ 351 (1965); *In re Sichert*, 566 F.2d 1154, 1159, 196 USPQ 209, 212-13 (CCPA 1977).

Compliance with 35 U.S.C. § 101 is a question of fact. *Raytheon v. Roper*, 724 F.2d 951, 956, 220 USPQ 592, 596 (Fed. Cir. 1983) cert. denied, 469 US 835 (1984). According to the PTO Utility Examination Guidelines (2001), irrefutable proof of a claimed utility is not required. Rather, a specific, substantial, and credible utility requires only a "reasonable" confirmation of a real world context of use. The evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the totality of the evidence under consideration. *In re Oetiker*, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992). The M.P.E.P. § 2107 clearly state that "[w]here the asserted utility is not specific or substantial, a *prima facie* showing must establish that it is more likely than not that a person of ordinary skill in the art would not consider that any utility asserted by the applicant would be specific and substantial." The M.P.E.P. also states that the *prima facie* showing must contain the "[s]upport for the factual findings relied upon" in reaching the conclusion that the utility is not substantial and specific. Thus, the initial burden is on the PTO to establish that it is more likely than not that one of skill in the art would not consider the asserted utility substantial, and that conclusion must be supported. Only after the PTO has made a properly supported *prima facie* showing of lack of utility does the burden of rebuttal shift to the applicant. The issue will then be decided on the totality of evidence, with the applicant needing to provide a "reasonable" confirmation of a real world context of use by a preponderance of the totality of the evidence.

Those of Skill in the Art Have Long Recognized a Strong Correlation Between Gene Amplification and Increased Protein Levels

The teachings in Genes V, a leading textbook in the field, illustrate that at the time the instant application was filed, it was well known by those of skill in the art that gene amplification leads to overexpression of the corresponding gene product. (Benjamin Lewin, Genes V, 5th ed. 1994, pages 1196-1201, submitted herewith as Exhibit 1). In a section entitled "Insertion, translocation, or amplification may activate proto-oncogenes", the text describes various molecular events that lead to overexpression of a gene product, using the *c-myc* gene as an example. The first mechanism taught is insertion of a retrovirus upstream of the gene which causes it to be driven by a more efficient promoter, resulting in increased mRNA and protein levels. Next, Lewin teaches that chromosomal translocations may bring genes to a new region

where it is actively expressed, resulting in increased gene and protein expression. The third mechanism whereby protein levels of oncogenes are overexpressed is gene amplification. The text emphasizes that the common thread among the different means of activation of proto-oncogenes is that the expression of the gene is increased. Thus, as of 1994, it was well-known in the art that gene amplification is correlated with overexpression of the corresponding mRNA and encoded protein.

Additional information regarding the understanding of those of skill in the art regarding the relationship between gene amplification and protein overexpression at the time the instant application was filed is found in Alitalo (Med. Biol.,. 62:304-317 (1984), submitted herewith as Exhibit 2), and Merlino *et al.* (J. Clin. Invest., 75:1077-1079 (1985), submitted herewith as Exhibit 3). Alitalo teaches gene amplification of oncogenes results in elevated expression of the gene, and that the increased dosage of the gene product may contribute to the progression of some cancers. Merlino *et al.* studied epidermoid carcinoma cells, and teach that amplification of the EGF receptor gene results in increased levels of EGF receptor mRNA and increased levels of EGF receptor protein. Taken together, the excerpt from Genes V, as well as the Alitalo and Merlino references establish that as of the filing date of the instant application, those of skill in the art appreciated the correlation between gene amplification and overexpression of the encoded gene product.

The teachings of Genes V, Alitalo, and Merlino are confirmed in several more recent reports that also document the correlation between gene amplification and levels of protein. Applicants submit herewith two more recent studies providing evidence that the teachings referred to above are still widely accepted by those of skill in the art. Bahnassy *et al.* (BMC Gastroenterology, 4:22-34 (2004), submitted herewith as Exhibit 4) studied the amplification of *cyclin D1, cyclin A, histone H3* and *Ki-67*, and assessed the levels of the encoded proteins by immunohistochemistry. Bahnassy *et al.* found a "significant correlation between *cyclin D1* gene amplification and protein overexpression" (Bahnassy at 27, column 1). Similarly, Blancato *et al.* (British Journal of Cancer, 90(8), 1612-1619 (2004), submitted herewith as Exhibit 5), report that overexpression of *c-myc* mRNA and c-Myc protein is related to the copy number of the *c-myc* amplification (Blancato at 1613, column 2). Bahnassy and Blancato demonstrate continued evidentiary support for the widely-accepted principle that gene amplification correlates with overexpression of the encoded protein.

Those of Skill in the Art Recognize a Strong Correlation Between Increased mRNA Levels and Increased Protein Levels

Applicants next address the Examiner's assertion that there is no evidence supporting Dr. Polakis' testimony that increased mRNA levels are predictive of increased levels of the encoded polypeptide. As the Examiner correctly pointed out, the data in Example 16 of the specification addresses gene amplification. Nevertheless, Applicants submit that it is well known that gene amplification is correlated with increased mRNA levels. See, e.g. Genes V, pages 1196-1201. Further, Applicants submit that it is well recognized that increased mRNA levels are in turn correlated with increased levels of the gene product. Applicants submit herewith an excerpt from Molecular Biology of the Cell, a leading textbook in the field (Bruce Alberts, *et al.*, Molecular Biology of the Cell (4th ed. 2002) submitted herewith as Exhibit 6), in support the previously submitted testimony of Polakis and Grimaldi regarding the correlation between mRNA levels and protein levels. Figure 6-3 on page 302 illustrates the basic principle that there is a correlation between increased gene expression and increased protein expression. The accompanying text states that "a cell can change (or regulate) the expression of each of its genes according to the needs of the moment – *most obviously by controlling the production of its mRNA*." Id. at 302, emphasis added. Similarly, figure 6-90 on page 364 illustrates the path from gene to protein. The accompanying text states that while potentially each step can be regulated by the cell, "**the initiation of transcription is the most common point for a cell to regulate the expression of each of its genes**." Id. at 364. This point is repeated on page 379, where the authors state that of all the possible points for regulating protein expression, "**[f]or most genes transcriptional controls are paramount**." Id. at 379. In short, Applicants have provided evidence in the form of excerpts from leading textbooks in the field, and several references which demonstrate that those of skill in the art appreciate the correlation between gene amplification, gene overexpression, and protein overexpression. The evidence submitted is sufficient to establish that more likely than not, one of skill in the art would accept the truth of Applicants' asserted utilities for PRO1800 based on the gene amplification data set forth in the specification.

Conclusion

Applicants have established that it is the general, accepted understanding in the art that gene amplification leads to increased gene and protein expression. Of particular significance is

the fact that the statements in the Genes V excerpt, as well as the general statements in the references submitted herein, have identified the general understanding in the field regarding the correlation between gene amplification and overexpression of the gene and gene product. Applicants respectfully submit that the totality of the above-cited evidence clearly establishes that those of skill in the art would believe that gene amplification leads to increased gene expression, and that mRNA levels more likely than not correlate with protein levels. In light of the fact that Applicants need not show a *necessary* correlation between gene amplification, mRNA and protein levels, Applicants respectfully submit that they have rebutted any prima facie case of non-utility and non-enablement the Examiner may have established. Accordingly, Applicants request withdrawal of the rejection of Claims 22-27.

Rejection under 35 U.S.C. § 112, first paragraph – Enablement

The Examiner maintains the rejection of Claims 27 and 32-35 under 35 U.S.C. § 112, first paragraph, as not being enabled by the specification, due to lack of utility of the claimed polypeptides. Applicants submit that for the reasons set forth above, PRO1800 polypeptides have credible, substantial, and specific utility. Thus, Applicants respectfully request that the Examiner reconsider and withdraw the rejections under 35 U.S.C. § 112, first paragraph.

CONCLUSION

In view of the above, Applicants respectfully maintain that the claims are patentable and request that they be passed to issue. Applicants invite the Examiner to call the undersigned if any remaining issues may be resolved by telephone.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

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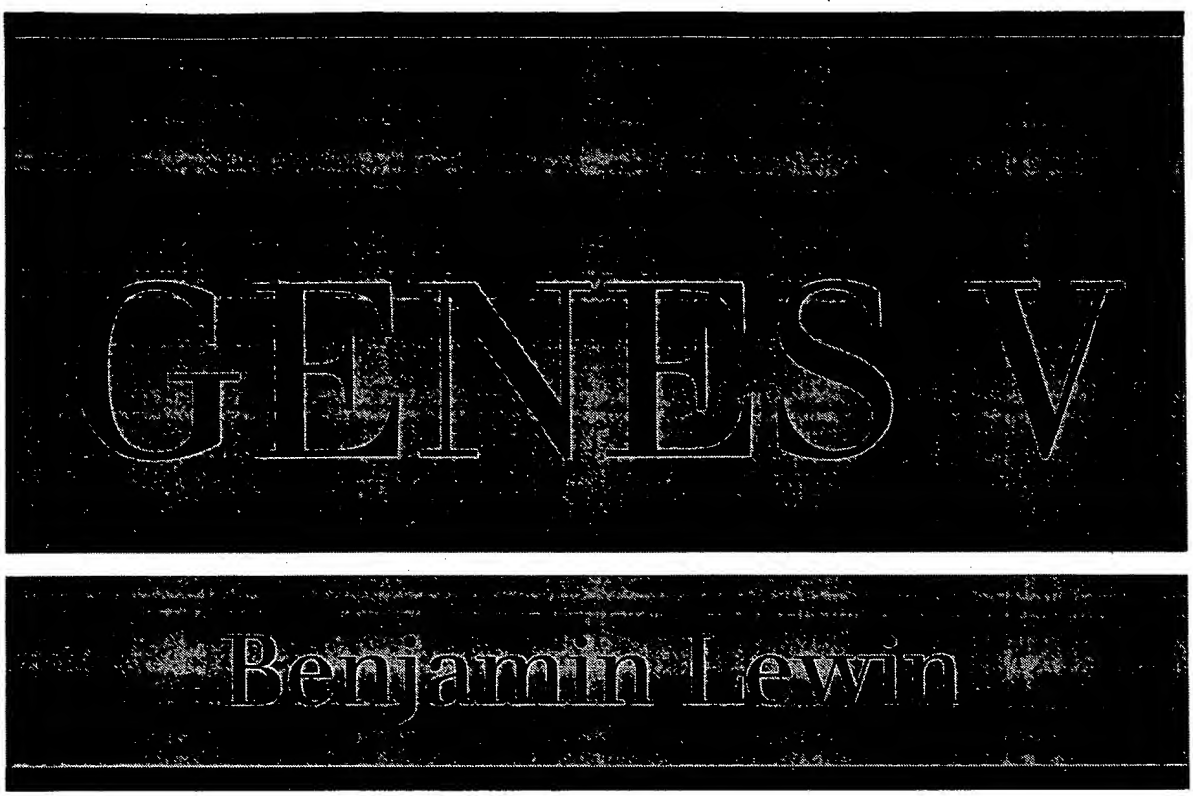

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~10×). Also, some tumor lines have amplified *ras* genes. A 20-fold increase in the level of a nontransforming Ras protein is sufficient to allow the transformation of some cells. The effect has not been fully quantitated, but it suggests the general conclusion that oncogenesis depends on overactivity of Ras protein, and is caused either by increasing the amount of protein or (probably more efficiently) by a variety of mutations that increase the activity of the protein.

Transfection by DNA can be used to transform only certain cell types. Although transforming oncogenes have been isolated from both rodent and human cells, most targets for transformation by transfection with oncogenes have been rodent fibroblasts in culture. (In fact, the difference in the source of the oncogene [human] and the recipient cell [rodent] is an important factor in allowing the donor gene to be distinguished unequivocally from recipient DNA.) Limitations of the assay explain why relatively few oncogenes have been detected by transfection. This system has been most effective with *ras* genes, where there is extensive correlation between mutations that activate *c-ras* genes in transfection and the occurrence of tumors.

Insertion, translocation, or amplification may activate proto-oncogenes

A variety of genomic changes can activate proto-oncogenes, sometimes involving a change in the target gene itself, sometimes activating it without changing the protein product. In cases of insertion and translocation, there is evidence that the genomic change is the causative event; in cases of amplification there is a correlation with tumorigenesis, but no direct proof for a causative role.

Many tumor cell lines have visible regions of chromosomal amplification, as shown by homogeneously staining regions (see Figure 36.27) or double minute chromosomes (see Figure 36.28). In some cases, the amplified region contains a known oncogene or a gene related to one. In other cases, where amplification is not visible, the use of batteries of probes representing oncogenes shows that a particular oncogene is amplified. Examples of oncogenes that are amplified in various tumors include *c-myc, c-abl, c-myb, c-erbB, and c-K-ras.*

Established cell lines are prone to amplify genes (it is one of several karyotypic changes to which they are susceptible). All the same, the presence of known oncogenes in the amplified regions, and the consistent amplification of particular oncogenes in many independent tumors of the same type, again strengthens the correlation between increased expression and tumor growth. Of course, it is possible that the gene amplification gives an advantage to growth of the established tumor; it is not necessarily an event involved in its initiation.

Some proto-oncogenes are activated by events that change their expression, but which leave their coding sequence unaltered. The best characterized is *c-myc,* whose expression is elevated by several mechanisms. One common mechanism is the insertion of a nondefective retrovirus in the vicinity of the gene.

The ability of a retrovirus to transform without expressing a *v-onc* sequence was first noted during analysis of the bursal lymphomas caused by the transformation of B lymphocytes with avian virus. Similar events occur in the induction of T cell lymphomas by murine leukemia virus. In each case, the transforming potential of the retrovirus seems to lie with its LTR rather than with a coding sequence.

In many independent tumors, the virus has integrated into the cellular genome within or close to the *c-myc* gene. The gene consists of three exons; the first represents a long nontranslated leader, and the second two code for the c-Myc

protein. **Figure 39.8** summarizes the types of insertion at this locus. The retrovirus may be inserted at a variety of locations relative to the *c-myc* gene.

The simplest insertions to explain are those that occur within the first intron. The LTR provides a promoter, and transcription reads through the two coding exons. Transcription of *c-myc* under viral control differs in two ways from its usual expression: the level of expression is increased (because the LTR provides an efficient promoter); and the transcript lacks its usual nontranslated leader (which may usually limit expression).

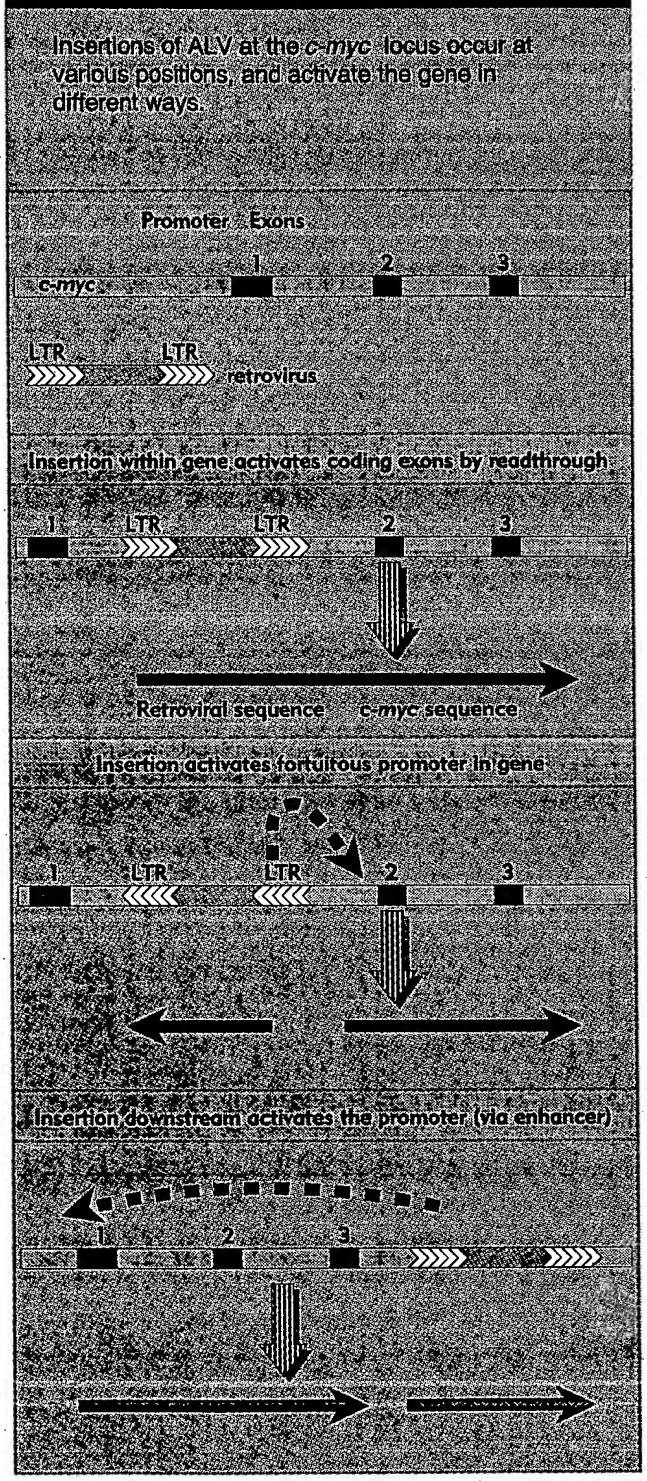
Activation of *c-myc* in the other two classes of insertions reflects different mechanisms. The retroviral genome may be inserted within or upstream of the first intron, but in reverse orientation, so that its promoter points in the wrong direction. Probably the LTR provides an enhancer that acts on an upstream sequence that fortuitously resembles a promoter. The retroviral genome also may be inserted downstream of the *c-myc* gene, in which case transcription initiates at the usual *c-myc* promoter(s), but is increased by the enhancer in the retroviral LTR.

In all of these cases, the coding sequence of c-myc *is unchanged, so oncogenicity is attributed to the loss of normal control, and increased expression, of the gene.*

Other oncogenes that are activated in tumors by the insertion of a retroviral genome include *c-erbB, c-myb, c-mos, c-H-ras,* and *c-raf.* Up to 10 other cellular genes (not previously identified as oncogenes by their presence in transforming viruses) are implicated as potential oncogenes by this criterion. The best characterized among this latter class are *wnt1* and *int2.* The *wnt1* gene codes for a protein involved in early embryogenesis that is related to the *wingless* gene of *Drosophila; int2* codes for a growth factor related to FGF.

Translocation to a new chromosomal location is another of the mechanisms by which oncogenes are activated. Certain chromosomal translocations are consistently associated with activation of oncogenes that lie near the breakpoints. This situation was originally discovered via a connection between the loci coding immunoglobulins and the

Figure 39.8



occurrence of certain tumors. Specific chromosomal translocations are often associated with plasmacytomas in the mouse and with Burkitt lymphomas in man. These tumors arise from undifferentiated B lymphocytes. The common feature in both species is that an oncogene on one chromosome is brought into the proximity of an Ig locus on another chromosome. Similar events occur in T lymphocytes to bring oncogenes into the proximity of a TcR locus.

The basic cause of the translocation event is a malfunction of the system responsible for recombining V and C genes or switching between IgH C genes. Instead of acting on two sites within the Ig or TcR locus, the system recombines the immune locus with an unrelated region on a different chromosome. This results in a **reciprocal translocation**, which is illustrated in **Figure 39.9**.

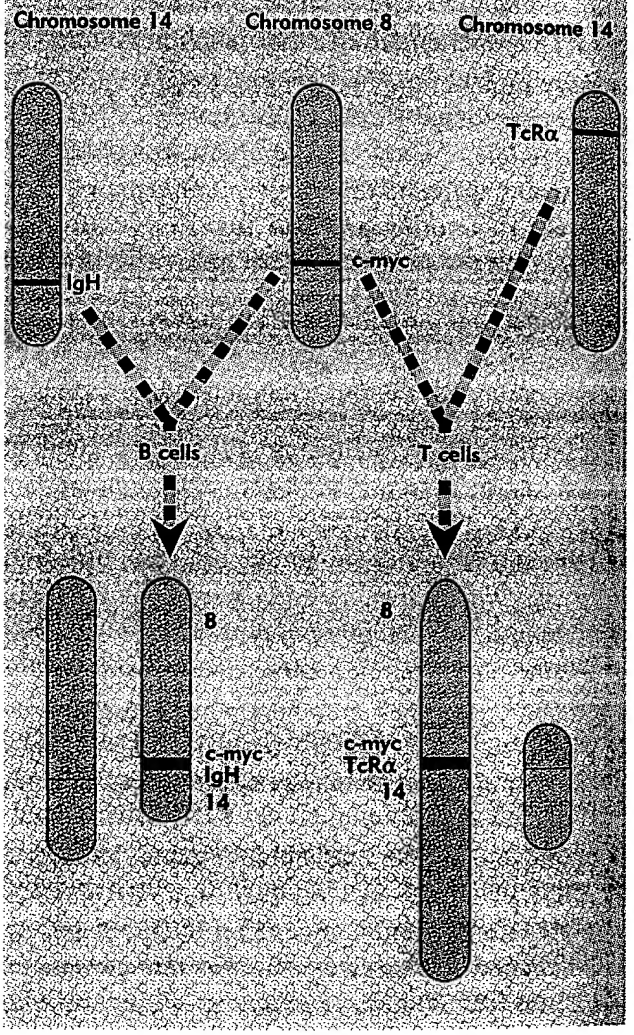
We do not know the basis for the involvement of the nonimmune partner, but in both man and mouse it is often the *c-myc* locus. In man, the translocations in B cell tumors usually involve chromosome 8, which carries *c-myc,* and chromosome 14, which carries the IgH locus; ~10% involve chromosome 8 and either chromosome 2 (κ locus) or chromosome 22 (lambda locus). The translocations in T cell tumors often involve chromosome 8, and either chromosome 14 (which has the TcRα locus at the other end from the Ig locus) or chromosome 7 (which carries TcR β locus). Analogous translocations occur in the mouse.

Translocations at the IgH locus in B cells fall into two classes. One type is similar to those observed at other Ig loci and at TcR loci, involving the consensus sequences used for V-D-J somatic recombination of active Ig genes. In the other type, the translocation occurs at a switching site, so these cases may be associated with function of the system that switches expression from one C_H gene to another.

When *c-myc* is translocated to the Ig locus, its level of expression is usually increased. The increase varies considerably among individual tumors, generally being in the range from 2 to 10. Why does translocation activate the *c-myc* gene? The translocation event does not involve fixed sites,

Figure 39.9

A chromosomal translocation is a reciprocal event that exchanges parts of two chromosomes. Translocations that activate the human *c-myc* proto-oncogene involve Ig loci in B cells and TcR loci in T cells.



but occurs at a variety of locations within a general region on each recombining chromosome. The event has two consequences: *c-myc* is brought into a new region, one in which an Ig or TcR gene was actively expressed; and the structure of the *c-myc* gene may itself be changed (but usually not involving the coding regions). It seems likely that several different mechanisms can activate the *c-myc* gene

in its new location (just as retroviral insertions activate *c-myc* in a variety of ways).

The correlation between the tumorigenic phenotype and the activation of *c-myc* by either insertion or translocation suggests that continued high expression of the c-Myc protein is oncogenic. Expression of *c-myc* must be switched off to enable immature lymphocytes to differentiate into mature B and T cells; failure to turn off *c-myc* maintains the cells in the undifferentiated (dividing) state. The oncogenic potential of *c-myc* has been demonstrated directly by the creation of transgenic mice carrying a normal *c-myc* gene linked to an enhancer. Transgenic mice carrying a *c-myc* gene linked to a B lymphocyte-specific enhancer (the IgH enhancer) develop lymphomas. The tumors represent both immature and mature B lymphocytes, suggesting that over-expression of *c-myc* is tumorigenic throughout the B cell lineage. Transgenic mice carrying a *c-myc* gene under the control of the LTR from a mouse mammary tumor virus, however, develop a variety of cancers, including mammary carcinomas. This suggests that increased or continued expression of *c-myc* transforms the type of cell in which it occurs into a corresponding tumor. Specificity of the tumor type may therefore depend on the mechanism used to activate *c-myc*; it is not an intrinsic property of the gene.

c-myc exhibits three means of oncogene activation: retroviral insertion, chromosomal translocation, and gene amplification. The common thread among them is increased expression of the oncogene rather than a qualitative change in its coding function, although in at least some cases the transcript has lost the usual (and possibly regulatory) nontranslated leader. *c-myc* provides the paradigm for oncogenes that may be effectively activated by increased (or possibly altered) expression.

Every translocation generates reciprocal products; sometimes a known oncogene is activated in one of the products, but in other cases it is not evident which of the reciprocal products has responsibility for oncogenicity. Also, it is not axiomatic that the gene(s) at the breakpoint have responsibility; for example, the translocation could provide an enhancer that activates another gene nearby.

A variety of translocations found in B and T cells have identified new oncogenes. In some cases, the translocation generates a hybrid gene, in which an active transcription unit is broken by the translocation. This has the result that the exons of one gene may be connected to another. In such cases, there are two potential causes of oncogenicity: the proto-oncogene part of the protein may be activated in some way that is independent of the other part, for example, because it is over-expressed under its new management (a situation directly comparable with the example of *c-myc*); or the other partner in the hybrid gene may have some positive effect that generates a gain-of-function in the part of the protein coded by the proto-oncogene.

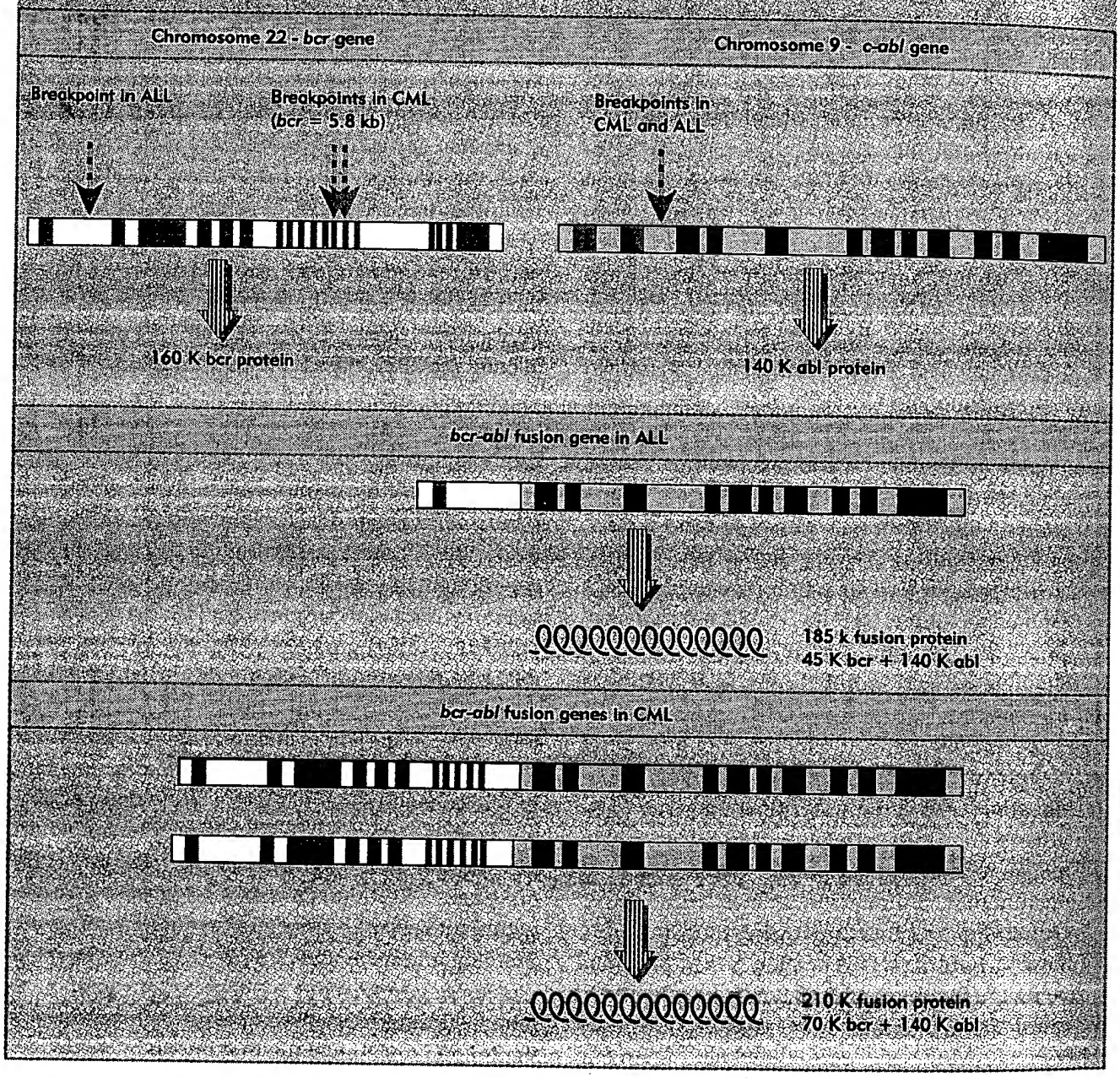
One of the best characterized cases in which a translocation creates a hybrid oncogene is provided by the *Philadelphia (PH¹)* chromosome present in patients with chronic myelogenous leukemia (CML). This reciprocal translocation is too small to be visible in the karyotype, but links a 5,000 kb region from the end of chromosome 9 carrying *c-abl* to the *bcr* region of chromosome 22. The *bcr* (*b*reakpoint *c*luster *r*egion) was originally named to describe a region of ~5.8 kb within which breakpoints occur on chromosome 22. Different cases of CML have breakpoints at different locations within this region.

The consequences of this translocation are summarized in **Figure 39.10**. The *bcr* region lies within a large (>90 kb) gene, which is now known as the *bcr* gene. The breakpoints in CML usually occur within one of two introns in the middle of the gene. The same gene is also involved in translocations that generate another disease, ALL (acute lymphoblastic leukemia); in this case, the breakpoint in the *bcr* gene occurs in the first intron.

The *c-abl* gene is expressed by alternative splicing that uses either of the first two exons. The breakpoints in both CML and ALL occur in the intron that precedes the first common exon. Although the exact breakpoints on both chromosomes 9 and 22 vary in individual cases, the

Figure 39.10

Translocations between chromosome 22 and chromosome 9 generate Philadelphia chromosomes that synthesize *bcr-abl* fusion transcripts that are responsible for two types of leukemia.



common outcome is the production of a transcript coding for a Bcr–Abl fusion protein, in which N-terminal sequences derived from *bcr* are linked to *c-abl* sequences. In ALL, the 185,000 dalton fusion protein has ~45,000 daltons of the Bcr protein linked to c-Abl; in CML the fusion protein of 210,000 daltons has ~70,000 daltons of the Bcr protein. In each case, the fusion protein contains ~140,000

daltons of the usual ~145,000 c-Abl protein, that is, it has lost just a few N-terminal amino acids of the c-Abl sequence.

Why is the fusion protein oncogenic? It relies on an interaction between the N-terminal region provided by *bcr* with the c-Abl protein. The *bcr* gene has a variety of sequence motifs related to proteins involved in signalling pathways, but the pertinent one is a serine/threonine kinase activity that is coded within the first exon. This autophosphorylates residues in this part of the protein, and the phosphorylation confers the ability to interact with a region of the c-Abl protein called the SH2 domain (we discuss the nature of SH2 domains later). This raises the possibility that the Bcr part of the protein interacts with the c-Abl sequences, perhaps changing their conformation and activating a latent oncogenic potential.

Changes at the N-terminus are involved in the activation of oncogenic activity of *v-abl*, a transforming version of the gene carried in a retrovirus. The *c-abl* gene codes for a tyrosine kinase activity; this activity is essential for transforming potential in oncogenic variants. Deletion (or replacement) of the N-terminal region activates the kinase activity and transforming capacity. So the N-terminus provides a domain that usually regulates kinase activity; its loss may cause inappropriate activation.

Loss of tumor suppressors causes tumor formation

The common theme in the role of oncogenes in tumorigenesis is that increased or altered activity of the gene product is oncogenic. Whether the oncogene is introduced by a virus or results from a mutation in the genome, it is dominant over its allelic proto-oncogene(s). A mutation that activates a single allele is tumorigenic. Tumorigenesis then results from gain of a function.

Certain tumors are caused by a different mechanism: loss of both alleles at a locus is tumorigenic. Propensity to form such tumors may be inherited through the germline; it also occurs as the result of somatic change in the individual. Tumorigenesis then results from loss of function. Such cases identify tumor suppressors: genes whose products are needed for normal cell function, and whose loss of function causes tumors. The two best characterized genes of this class code for the proteins RB and p53.

Retinoblastoma is a human childhood disease, involving a tumor on the retina. It occurs both as a heritable trait and sporadically (by somatic mutation). It is often associated with deletions of band q14 of human chromosome 13. The *RB* gene has been localized to this region by molecular cloning.

Figure 39.11 illustrates the situation. Retinoblastoma arises when both copies of the *RB* gene are inactivated. In the inherited form of the disease, one parental chromosome carries an alteration in this region, usually a deletion. A somatic event in retinal cells that causes loss of the other copy of the *RB* gene causes a tumor. In the sporadic form of the disease, the parental chromosomes are normal, and both *RB* alleles are lost by (individual) somatic events.

Almost half the cases of retinoblastoma show deletions at the *RB* locus. In other cases, transcripts of the locus are either absent or altered in length. The protein product is absent from retinoblastoma cells. The cause of the tumor is therefore loss of protein function, usually resulting from mutations that prevent gene expression (as opposed to point mutations that affect function of the protein product). Loss of *RB* is involved also in other forms of cancer, including osteosarcomas and small cell lung cancers.

What is the molecular function of RB? It interacts with a variety of other proteins, including several tumor antigens: SV40 T antigen, adenovirus E1A, human papilloma virus E7. One possibility is that part of the oncogenicity of these proteins is due to

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AMPLIFICATION OF CELLULAR ONCOGENES IN CANCER CELLS

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ABSTRACT

Regulatory or structural alterations of cellular oncogenes have been implicated in the causation of various cancers. Oncogene alteration by point mutations can result in a protein product with strongly enhanced oncogenic potential. Aberrant expression of cellular oncogenes may be due to tumour-specific chromosomal translocations that dysregulate the normal functions of a proto-oncogene. Amplification of cellular oncogenes can also augment their expression by increasing the amount of DNA template available for the production of mRNA. It appears that amplification of certain oncogenes is a common correlate of the progression of some tumours and also occurs as a rare sporadic event affecting various oncogenes in different types of cancer. Amplified copies of oncogenes may or may not be associated with chromosomal abnormalities signifying DNA amplification: double minute chromosomes and homogeneously staining chromosomal regions. Amplified oncogenes, whether sporadic or tumour type-specific, are expressed at elevated levels, in some cases in cells where their diploid forms are normally silent. Increased dosage of an amplified oncogene may contribute to the multistep progression of at least some cancers.

KEY WORDS: CELLULAR ONCOGENES, GENE AMPLIFICATION, MULTISTEP CARCINOGENESIS, CLONAL SELECTION, KARYOTYPIC ABNORMALITIES, DOUBLE MINUTE CHROMOSOMES, HOMOGENEOUSLY STAINING CHROMOSOMAL REGIONS

DNA SEQUENCE AMPLIFICATION AND CYTOGENETIC ABNORMALITIES IN TUMOURS

Since its discovery in drug-resistant eukaryotic cells, somatic amplification of specific genes has been implicated in an increasing variety of adaptive responses of cells to environmental stresses (70, 79). Cytogenetic abnormalities, double minute chromosomes (dmin:s) associated with DNA amplification had already been discovered in tumour cells before the discovery of dmin:s and homogeneously staining chromosomal regions (HSR:s) in cells selected for drug-resistance (12, 24, 49, 50, 56). In metaphase spreads, dmin:s appear as small, spherical, usually paired chromosome — like structures that lack a centromere (Fig. 2). HSR:s stain with intermediate intensity throughout their length rather than with the normal pattern of alternating dark and light bands in both trypsin-Giemsa (Fig. 3A) and quinacrine dihydrochloride-stained preparations. Both kinds of abnormalities are occasionally found in metaphases of freshly isolated cancer cells but not of normal cells (8).

Dmin:s and HSR:s are apparently rare in tumour cells in vivo, although exact data are difficult to obtain since the abnormalities are easily missed in routine cytogenetic analysis (8, 42). Dmin:s and HSR:s have been described in most types of in vitro-cultured malignant tumour cells, with a notable frequency in neuroblastoma cell lines (11). Initial growth in cell culture apparently selects for tumour cells that contain either dmin:s or HSR:s. Moreover, in culture dmin:s are frequently lost, concomitant with the appearance of clonal populations of cells that have developed an HSR, suggesting that the two cytogenetic abnormaltities are alternative forms of gene amplification and that HSR:s may confer a selective advantage on cells over dmin:s (11, 70). It has been assumed that HSR:s can break down to firm dmin:s and that dmin:s can integrate into chromosomes to generate HSR:s (11, 23). Amplified genes may also occupy abnormally banding regions, ABR:s (51, 59). Experimental work on drug-resistant cells has shown that in the absence of a selection pressure (drug), dmin:s and the amplified genes that they contain are lost, whereas amplified DNA in the form of HSR:s is retained in the cells (71). This is explained by the fact that dmin:s are segregated unevenly in mitosis and frequently get lost from the nucleus due to

TABLE 1

Currently known oncogenes.

Retrovirus (example)	Oncogene	Gene product		
		Cellular location	Function of protein	Class
ONCOGENES FOUND IN RETROVIRUSES				
RSV	*src*	Plasma membrane	Tyrosine-specific protein kinases (*fgr* contains sequences homologous to actin)	Class Ia (Cytoplasmic tyrosine protein kinases)
Y73V	*yes*	Plasma membrane		
GR-FeSV	*fgr*	Plasma membrane		
Ab-MuLV	*abl*	Plasma membrane		
FuSV	*fps/fes*	Cytoplasm (plasma membrane?)		
ST-and GA-FeSV	*fes/fps*	Cytoplasm (cytoskeleton?)		
UR2V	*ros*			
AEV	*erb*-B	Plasma membrane and cytoplasmic membranes	EGF receptor's cytoplasmic domain	Class 1b (Class 1a-related proteins)
SM-FeSV	*fms*	Plasma membrane and cytoplasmic membranes	Cytoplasmic domain of a growth factor receptor?	
MH-2V	*mil/raf*	Cytoplasm	?	
3911-MSV	*raf/mil*	Cytoplasm	?	
Mo-MSV	*mos*	Cytoplasm	?	
SSV	*sis*	Secreted	PDGF-like growth factor	Class 2 (Growth factors)
Ha-MSV	Ha-*ras*	Plasma membrane	GTP-binding proteins	Class 3 (Cytoplasmic GTP:ases)
Ki-MSV	Ki-*ras*	Plasma membrane		
FBJ-MuSV	*fos*	Nucleus	?	Class 4 (Nuclear phosphoproteins)
OK-10V	*myc*	Nucleus	Nuclear matrix protein	
AMV	*myb*	Nucleus	?	
SKV 770	*ski*	Nucleus?	?	Unclassified
REV	*rel*	?	?	
AEV	*erb*-A	?	?	
E26V	*ets*	?	?	
ONCOGENES FOUND IN TUMOUR CELLS BUT NOT IN RETROVIRUSES				
Tumour cell				
Neuroblastoma	N-*ras*	Plasma membrane	GTP—binding	Class 3
Neuroblastoma	N-*myc*	?	?	Class 4
Small-cell lung cancer	L-*myc*	?	?	Class 4
Neuro-/Glioblastomas	*neu*	Plasma membrane	Growth factor receptor	Class 1b

their lack of centromeres, (49). HSR chromosomes carry centromeres and are therefore divided equally at mitosis. If dmin:s and HSR:s contain amplified genes that encode growth-stimulating protein products, it would follow that the more stable chromosomal form, the HSR, confers a greater selective growth advantage for cells. Although dmin:s and HSR:s have been described predominantly in tumour cells selected for resistance to cytotoxic drugs, it is also clear that dmin:s and HSR:s may be present in cancer cells before any form of therapy (8). It was in this setting that we and others first chose to explore the possible amplification of cellular oncogenes. (By definition, cellular oncogenes are normally innocent genetic loci which can be activated to transforming genes in various ways).[1]

DMIN:S AND HSR:S CONTAIN AMPLIFIED ONCOGENES

Table 2 summarizes the somatic amplifications of cellular oncogenes so far reported in

[1] It is not the purpose of this review to deal with all forms of DNA damage that have been found to activate cellular oncogenes. For the purpose of integrating the review into a coherent picture, however, the reader is given a list of known cellular oncogenes in Table 1 and the schematic Figure 1 illustrating the various ways in which the oncogenic potential of different proto-oncogenes can be activated.

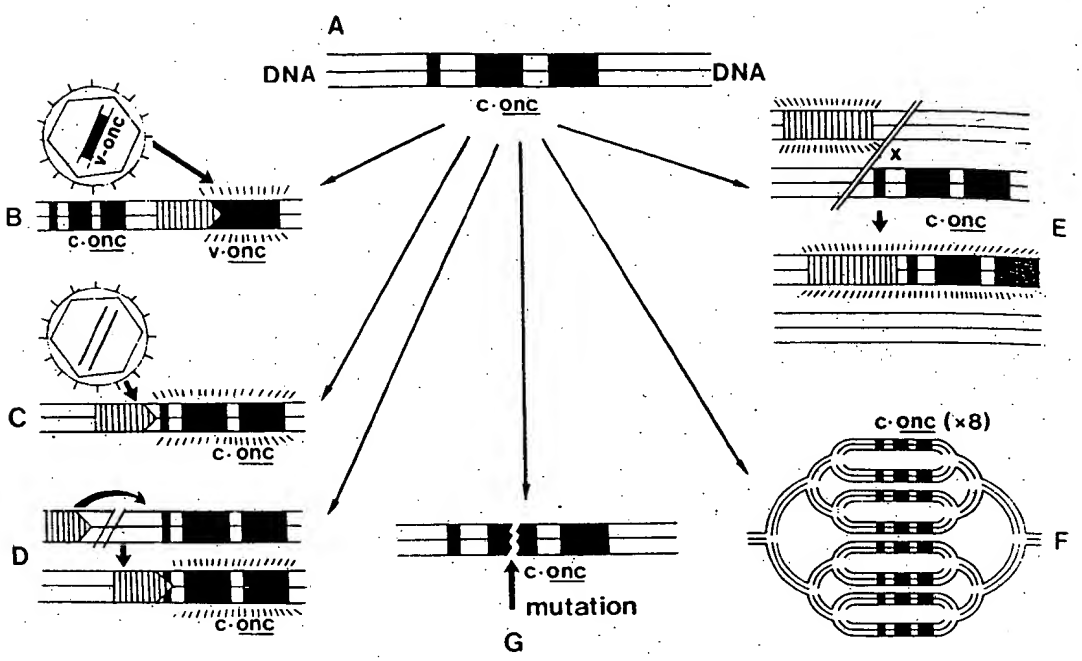

Fig. 1. Activation of cellular oncogenes. The haploid complement of a proto-oncogene is schematically depicted in A, composed of three exons (black boxes) in a segment of DNA. The different activated forms are schematically outlined in B—G. The abbreviation c-*onc* stands for cellular oncogene, v-*onc* viral oncogene, DNA sequences with associated strong promoter/enhancer functions are striated, and an actively transcribed gene is marked with radiations. B. Acute transforming retroviruses have the capacity to transduce cellular oncogenes (c-*onc*) into their genome, modify them and reinsert their activated oncogenes (v-*onc*) into the genome of host animal cells as a part of their proviral forms. The activity of the v-*onc* gene is greatly enhanced due to the associated promoter of the proviral long terminal repeat. Both increased dosage of the oncogene and structural mutations within its sequence may contribute to tumorigenesis. C. Slow transforming retroviruses without oncogenes replicate and reinsert their proviral copies into the host cell DNA during a latency period from infection to tumorigenesis. Tumor initiation through hyperplastic growth may begin, when the provirus integrates sufficiently close to a proto-oncogene to activate it through promoter or enchancer functions. It should be noted, however, that mutations have also been found in the oncogenes thus activated and that mutational damage to other oncogenes has been described in the resulting tumors. D. In some mouse plasmacytomas, a retrovirus-like DNA element (directing the synthesis of the so-called intracisternal A-type particles, IAPs) has been found in association with a transcriptionally activated oncogene c-*mos*. The IAP insertion also disrupts the 5' part of c-*mos* (64). E. In humans, as well as in animals, chromosome translocations may place proto-oncogenes into transcriptionally active regions of chromatin, where they may be activated. The details of this mechanism have not been worked out, but it is believed to occur for c-*myc* and c-*abl* genes in Burkitt lymphomas and Philadelphia-chromosome positive leukemias, respectively (35, 40). F. Increased amounts of oncogene-specific RNA and protein can also result from an excess of DNA template for transcription acquired through oncogene amplification. The present review concentrates primarily on this mechanism. G. Mutationally activated oncogenes have been found in nearly one fifth of human malignant tumours. Oncogene loci activated by somatic structural mutations are revealed by transfection experiments, where they are introduced into genetic background of nontumourigenic cultured immortalized cells. Several such transforming loci have been cloned and many of them belong to the c-*ras* oncogene family. It should be pointed out that both structural mutations and either increased expression or activation of a complementing oncogene may be required to achieve a fully tumorigenic phenotype (44).

tumour cells. Although the sampling of tumours is at present small, the finding of known cellular oncogenes among amplified DNA represented by dmin:s and HSR:s of cancer cells is provocative. Amplification has been found to affect at least five out of twenty known cellular oncogenes and the degree of gene amplification varies from five to many hundred-fold over the single haploid copies found in normal cells (see also ref. 18). The first amplification reported involved the c-*myc* oncogene (see Table 1) in a promyelocytic leukaemia cell line HL-60 (20, 25). The degree of c-*myc* amplification is between 8—32 fold both in the HL-60 cell line and in primary leukaemic cells from the patient (20, 25). Original clonal lines of HL-60 were later found to contain some dmin:s in culture but their number was insufficient to establish any clear correlation with amplified c-*myc*. Such a correlation, however, was discovered for c-*myc* amplification in a neuroendocrine cell line from

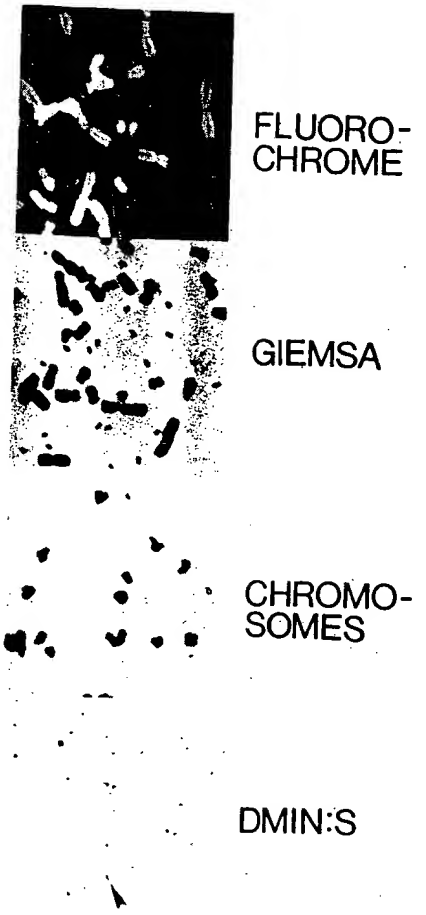

Fig. 2. Double minute chromosomes (arrowheads) in COLO 320DM colon carcinoma cells. A. The dmin:s are resolved as paired dots among normal chromosomes in this fluorescent, benzimidazole-stained preparation B—D. Purification of dmin:s by differential centrifugations. B. The starting material, C. Chromosome fraction, D. Purified dmin:s (Donna George and the author, unpublished data and ref. 52).

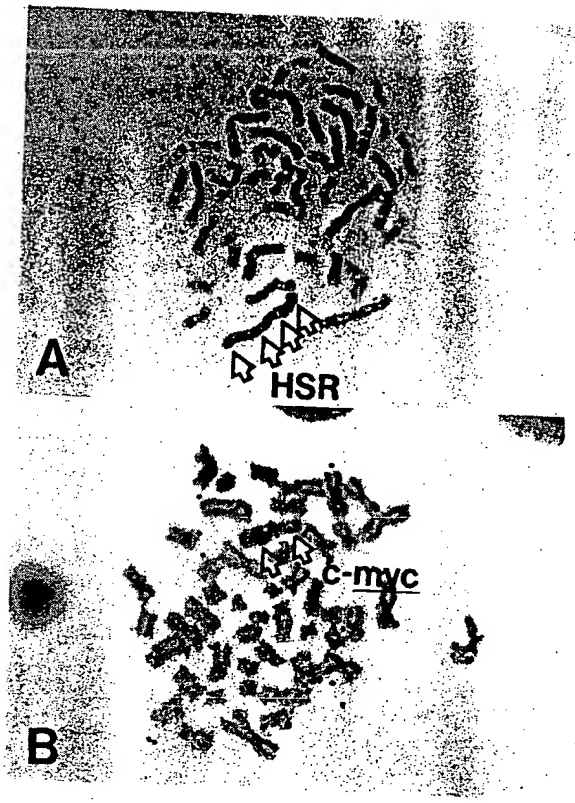

Fig. 3. A. The homogeneously staining regions (HSR) in the G-banded HSR-marker chromosome comprise a major portion of both its long and short arms. The HSR-marker chromosome has evolved from an X-chromosome (52 and unpublished data of C. C. Lin and the author). B. The about 30-fold amplified copies of the c-*myc* oncogene in COLO 320 cells were found to be located to dmin:s and HSR:s. The latter is shown here by in situ-hybridization (5, 52).

a colon carcinoma, COLO 320 (5). In these cells, the approximately 30-fold amplified c-*myc* copies were mapped either to HSR:s of a marker chromosome (5, Fig 3B) or to dmin:s (52), depending on the particular subline studied. Since dmin:s were already present in the primary tumour cells from this colon carcinoma (63), it is likely that c-*myc* had also been amplified during growth of the tumour in vivo. Similarly, amplified copies of the c-Ki-*ras* oncogene were mapped to dmin:s and HSR:s of a mouse adrenocortical tumor Y1 (74). An extensive search for changes in other oncogenes and tumour cells has since revealed amplifications that do not show up as dmin:s or HSR:s. Thus, for example, the c-*myb* oncogene is amplified in a characteristic marker chromosome of a colon carcinoma without evidence of HSR:s (ref. 6, Fig. 4) and in other tumours, the amplified c-*abl* and c-*myc* oncogene loci map to abnormally banding regions (ABR:s) in translocated or resident chromosomal segments, respectively (59, 76).

TRANSLOCATIONS AND REARRANGEMENTS MAY ACCOMPANY ONCOGENE AMPLIFICATION

The evolution and progression of the karyotype of tumour cells is complicated (see ref. 68). Concomitant with amplification, DNA sequences acquire an increased mobility in the genome with extrachromosomal intermediates

TABLE 2

Sporadic and tumour-specific amplification of cellular oncogenes. *

Tumour cells	Onco-gene	Fold amplified	Chromosomal location of amplified gene	Expression elevated	Remarks	References
Sporadic:						
HL60 (acute promyelocytic leukaemia, M3)	c-*myc*	20×	8q(ABR)	Yes	Amplification present in primary leukaemic cells	20, 25, 59
COLO320 (colon carcinoma)	c-*myc*	30×	dmin, HSR	Yes	Part of the amplified c-*myc* sequences rearranged	4, 5, 52
Y1 (adrenocortical tumour)	c-Ki-*ras*	50×	dmin, HSR	Yes	Levels of p $21^{c\text{-}Ki\text{-}ras}$ protein elevated	74
COLO201/205 (colon carcinoma)	c-*myb*	10×	mar1	Yes	Patient treated with 5-fluorouracil prior to culturing of the tumour cells	4, 6,88
K562 (chronic myelogenous leukaemia, CML)	c-*abl*	10×	mar(ABR)	Yes	C_λ coamplified in the marker that may be derived from chromosome 22, c-*abl* protein-associated tyrosine kinase activated	21, 22, 41, 54, 76
A431 (epidermoid carcinoma)	c-*erb*B	15—20×	n.d.	Yes	Amplification linked to chromosome 7 translocation and sequence rearrangements Amount of protein product, the EGF receptor, elevated	82 (see 36)
ML1—3 (acute myeloid leukaemia, M2)	c-*myb*	5—10×	n.d.	Yes	Abnormalities of chromosome 6q22—24, where c-*myb* is normally located	34, 61, 91
SK BR-3 (breast carcinoma)	c-*myc*	10×	n.d.	Yes		43
SEWA (polyoma virus-induced mouse tumour)	c-*myc*	30×	n.d.	Yes	Cells have dmin:s depending on culture conditions; c-*myc* amplification correlates with growth as a tumour	Manfred Schwab, personal communication
Lu-65 (lung giant cell carcinoma)	c-*myc* c-Ki-*ras*	8× 10×	n.d. n.d.	n.d. n.d.	At least some copies of c-Ki-*ras* mutated	80
Primary leukemic cells from an acute myeloid leukemia (M2) patient	c-*myc*	33×	n.d.	n.d.		Unpublished data of the author and A. de la Chapelle
Tumour-specific:						
small-cell lung cancer	c-*myc* L-*myc*, N-*myc*	up to 80×	n.d.	Yes	Most amplifications in the variant phenotype of SCLC	53, 69
Neuroblastomas	N-*myc*	up to 250×	dmin, HSR	Yes	N-*myc* also amplified in primary tumours of advanced grade	14, 48, 72, 73, 75
Glioblastomas	c-*erb*B	—	—	—	Rearrangements also found personal communication	Josef Schlessinger, personal communication

n.d. = not determined, mar = marker chromosome, M2, M3 refer to the French-American-Brittish classification of acute myeloid leukemias.

* At least one case of oncogene amplification in normal germ-line cells has been found (18).

visualized as dmin:s, transpositions and translocations to other chromosomal segments, etc. (see 70 for references). There may not be preferred chromosomal sites for the apparent reintegration of dmin:s as HSR:s (75). In at least one case, however, an oncogene may have been caught amplifying in situ in its resident chromosomal site (59). The finding of moderately amplified oncogenes also in chromosomal sites lacking HSR:s suggests that

Fig. 4. Localization of amplified c-*myb* in COLO 201/205 cells by in situ hybridization. Shown is a characteristic, large marker chromosome (mar1) with G-banding (A) and associated c-*myb* autoradiographic grains (B). Note the absence of HSR:s. Marl has probably evolved from chromosome number 6, the resident site of the c-*myb* oncogene in normal cells (34, 88, 91). (Robert Winqvist and the author, unpublished data).

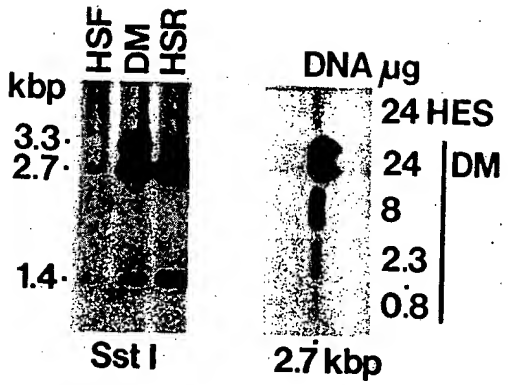

Fig. 5. Amplification and rearrangement of c-*myc* in COLO 320 cells. 10 µg of cellular DNA was digested with Sst I, electrophoresed, blotted and probed with a v-*myc* Pst I fragment (ref. 2, left panel). Fragments of 2.7 kbp and 1.4 kbp are seen in both normal and amplified c-*myc* DNA. The 3.3 kbp fragment is derived from a DNA segment of unknown origin translocated to the 5′ region of c-*myc* with a concomitant deletion of its first exon (unpublished data of Manfred Schwab and the author). HSF, human skin fibroblasts; DM, COLO 320 DM cells; HSR, COLO 320 HSR cells. Different amounts of DNA from COLO 320 DM cells as indicated were mixed with calf thymus DNA to give 24 µg of total DNA, cleaved with Sst I, electrophoresed, blotted and probed with a fragment of 3′human c-*myc* sequences. The intensities of the 2.7 kbp c-*myc* fragment in different samples were compared to assess its copy number, estimated to be about 30 (5).

(onco)gene amplification may be more common than the structural alterations shown by chromosome banding and microscopy (6, 88).

In at least three cases reported amplification has been accompanied by a DNA rearrangement of the oncogene (5, 20, 82). In the colon carcinoma COLO 320 both damaged and normal versions of the c-*myc* gene are amplified (5). Although individual cell clones have not yet been examined, our unpublished experiments suggest that the same dmin-containing cells harbor and express both normal and rearranged forms of c-*myc*. The normal version of the amplified gene, however, predominates in COLO 320 cells containing HSR:s; the rearranged version is present only in what appears to be a single copy (Fig. 5). In the chronic myeloid leukaemia (erythroleukaemia) cell line K562 an amplified DNA segment consists of portions of both the c-*abl* oncogene and the immunoglobulin C_λ locus (76). In both cases abnormal transcripts are produced from the rearranged amplified oncogenes (Fig. 6 and ref. 22). In K562 cells, the abnormal c-*abl* oncogene product has also been activated as a tyrosine protein kinase (41). It is not known, however, whether structural alterations of the genes preceded amplification or whether they were acquired during the process of gene amplification. It seems likely that a chromosomal translocation of c-*abl* to the C_λ locus preceded DNA amplification in the K562 cells, since all amplified copies were also rearranged (21), with the change reminiscent of the Philadelphia translocation (t(9, 22)) found in most chronic myeloid leukaemia tumours (35, 66—68). Although they have not been sequenced, other reported cases of amplified oncogenes are apparently normal on basis of mapping with restriction endonucleases (see Table 2). Therefore we cannot at present view mutation as a necessary companion of oncogene amplification.

THE MECHANISMS OF GENE AMPLIFICATION

The mechanisms of gene amplification and the structure of the amplified DNA have been worked out mainly in experimental settings involving selection for drug-resistance in cell culture (70). Although the mechanisms are still incompletely known and may vary in different cases, some general features have emerged.

A spontaneous degree of illegitimate DNA replication seems to exist in normal cells so that various segments of DNA are replicated more than once during a single cell cycle (37). In unselective conditions this DNA is probably lost e.g., through formation of micronucleae

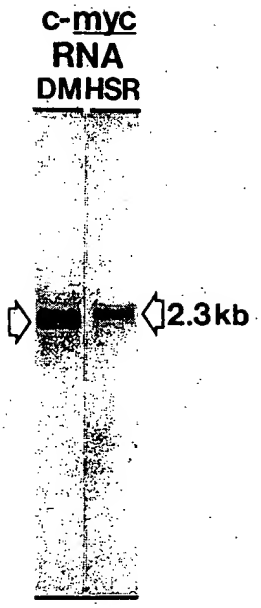

Fig. 6. Comparison of the electrophoretic mobilities of c-*myc* mRNA:s from COLO 320 DM and HSR cells. The size of the normal c-*myc* mRNA is 2.3 kb. The rearranged c-*myc* locus in DM cells (see Fig. 5) seems to be predominantly expressed giving rise to a shortened RNA.

because the newly synthesised extra copies of DNA are not covalently linked to chromosomal DNA of mitotic cells (65, 71). If, however, there is a selection pressure to retain an increased gene dosage, progressive multiplication of gene copy number results. The incidence of cells bearing amplified genes under conditions of cytotoxic selection can vary by two orders of magnitude and is greatly increased by the presence of mitogenic substances (hormones or tumour promoters) during selection (10, 84, 85) or certain carcinogenic or cytotoxic agents before selection (15, 55, 79, 80, 81, 85). An interesting hypothesis suggested by Varshavsky (84, 85) supposes that the origins of DNA replication "fire" (initiate replication) illegitimately several times during a single cell cycle and that this kind of "replicon misfiring" may be increased by substances such as tumour promoters and mitogenic hormones (10, 84, 85). Mariani and Schimke (55) point out that most of the cytotoxic agents that increase the incidence of gene amplification are inhibitors of DNA synthesis. Aberrant replication is known to take place after transient inhibition of DNA synthesis and this response can lead to gene amplification (46, 47, 55, 90). Mitogenic hormones probably increase disproportionate DNA replication, but they also enhance the colony forming efficiency of drug-resistant cells in selective conditions (10).

According to the studies of Axel and his collaborators (65), the multiple cycles of unscheduled DNA replication at a single locus during a single cell cycle result in a structure schematically outlined in Fig. 1F. The hydrogen-bonded amplified copies of DNA depicted in Fig. 1F must resolve into a tandem linear array before the next mitosis. This may well occur by homologous recombination between any one of several repeated sequences within the amplified domain (45, 65). Part of the recombinations would lead to extrachromosomal circles possessing an origin for replication (16, 62); these could be the precursors of dmin:s. The unequal recombinations mean that the resolved linear structure consists of tandemly repeated but heterogeneous units. According to Axel's model a gradient of amplification is formed so that centrally located sequences are amplified more than sequences distal to the origin of replication (65). This also has, in fact, been found to explain the large, complex DNA domain amplified in neuroblastoma cells in vivo (38, see also below).

The chromosomal site of integration of transfected genes significantly affects the frequency and cytogenetic result of their experimentally induced amplification (83). The amplification frequency in some transformants has been found to be 100-fold that of the others (83). This suggests that there also are preferred chromosomal positions for amplification of host cellular genes and that chromosomal rearrangements may facilitate gene amplification by positioning chromosomal sequences in a favorable array. In respect of the structural properties of the sequences involved in gene amplification, recombinatorially active regions have been implicated in experimental cases. DNA rearrangements involving restriction fragment length polymorphisms and variation in gene copy number have been detected in the human genome between clusters of short repetitive interspersed DNA sequences called Alu family DNA-sequences (17). Such inter-Alu sequences have also been detected in an extrachromosomal DNA form, including covalently closed circles (17, 78). The copy number of inter-Alu sequences apparently varies in an age- and tissue-specific manner (17, 78), but any comprehensive analysis of the phenomenon in human tumours is not yet available. It is also not yet clear whether these kinds of repetitive sequences are involved in generating amplified oncogene sequences in dmin:s or HSR:s in tumours.

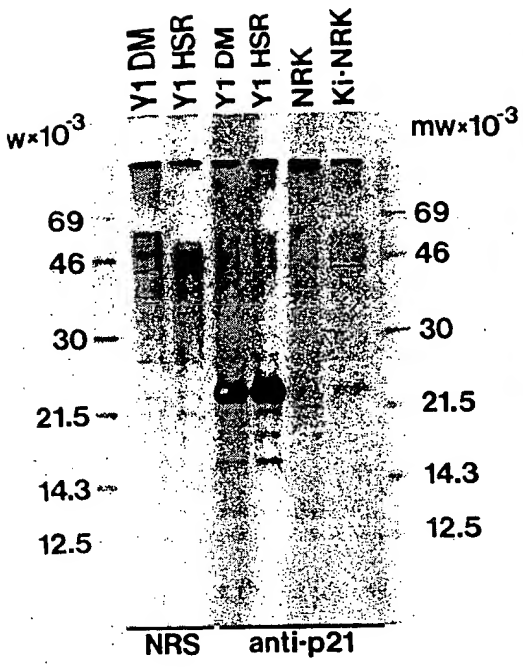

Fig. 7. Elevated levels of the p21$^{c\text{-}Ki\text{-}ras}$ protein in Y1 cells (74). The Y1 DM and HSR cells which harbor a 50-fold amplified c-Ki-*ras* oncogene (74) and control cells were labeled with [^{35}S]-methionine and the p21$^{c\text{-}Ki\text{-}ras}$ protein was immunoprecipitated, as detailed (74), with normal rat serum (NRS) or rat monoclonal anti-p21 serum. The proteins were electrophoresed in a 15 % polyacrylamide gel in the presence of SDS. In addition to a major p21 band, a labeled band at about 16 kd was present in the immunoprecipitates. The amount of radioactivity in p21 was about 50 fold that in normal rat kidney cells. The Kristen sarcoma virus-transformed rat kidney cells (obtained from the American Tissue Culture Collection) also yielded unexpectedly low amounts of the v-Ki-*ras* protein.

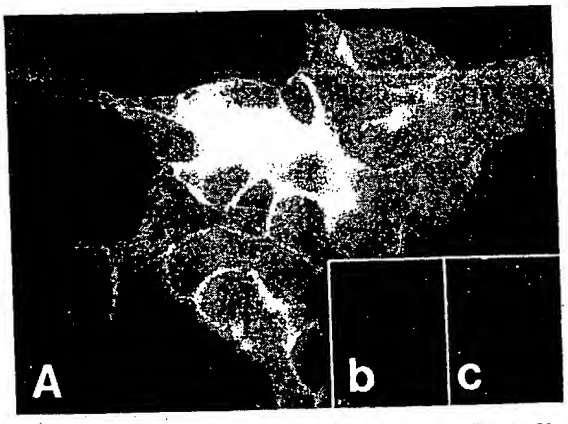

Fig. 8. A. Indirect immunofluorescence for p21$^{c\text{-}Ki\text{-}ras}$ in Y1 DM cells. Similar fluorescence of the plasma membrane was obtained for the Y1 HSR cells. Inset (b) shows control staining with normal rat serum and inset (c) staining of normal rat kidney cells with the monoclonal antibody against p21.

CARCINOGEN-INDUCED GENE AMPLIFICATION AND CLONAL SELECTION OF CANCER CELLS

Although cell sorting experiments have shown a basal spontaneous rate of gene amplification in eukaryotic cells (37), this can be increased severalfold by metabolic inhibitors or cytotoxic agents (15, 37, 70, 81, 85). In many respects the latter response is reminiscent of the so-called SOS-response elicited in bacteriae by noxious stimuli (see 28). In a teleological context, the rapid induction of gene amplification that apparently occurs frequently through extrachromosomal intermediates may provide cells with genetic material for subsequent selective pressures operating in harmful conditions (60). In cancer cells, the mechanism may enhance the emergence of clonal populations of cells with increasingly malignant properties (58, 60). Such genetic instability of cancer cells is clearly enhanced, leading to the rapid evolution of increasingly malignant tumour cell populations (19, 58). A serious question of practical importance is whether drug resistance in treated patients also selects cells that have an enhanced ability to amplify (onco)genes important for growth and progression of the tumour (84, 85). It is also possible that some of the carcinogenic insults caused by mutagens are only expressed as a result of subsequent amplification events induced by tumour promoters (84, 85) or facilitated by hormones in, say replicating epithelial cells (10). The persistence of dmin:s in some tumours suggests that there is a selection pressure for their retention (8, 9, 11, 23). Amplified DNA in dmin:s must contain an origin for DNA replication (62) and must be selected for in daughter cell populations, where it is unevenly segregated (71). In the absence of such a selection pressure dmin:s are lost (71). In at least one study the length of an HSR has been found to increase during a selection of malignant cells for enhanced tumourigenicity (30).

The amplified c-*erb*B gene in A431 cells codes for epidermal growth factor receptor (27). The abundant amounts of receptor protein on A431 cell surface may, however, provide the cells with an abnormal growth response (31). A naive supposition is that the amplified sequences in dmin:s and possibly in HSR:s of tumours contain growth-promoting genes (see 36 for references). This seems to fit well with

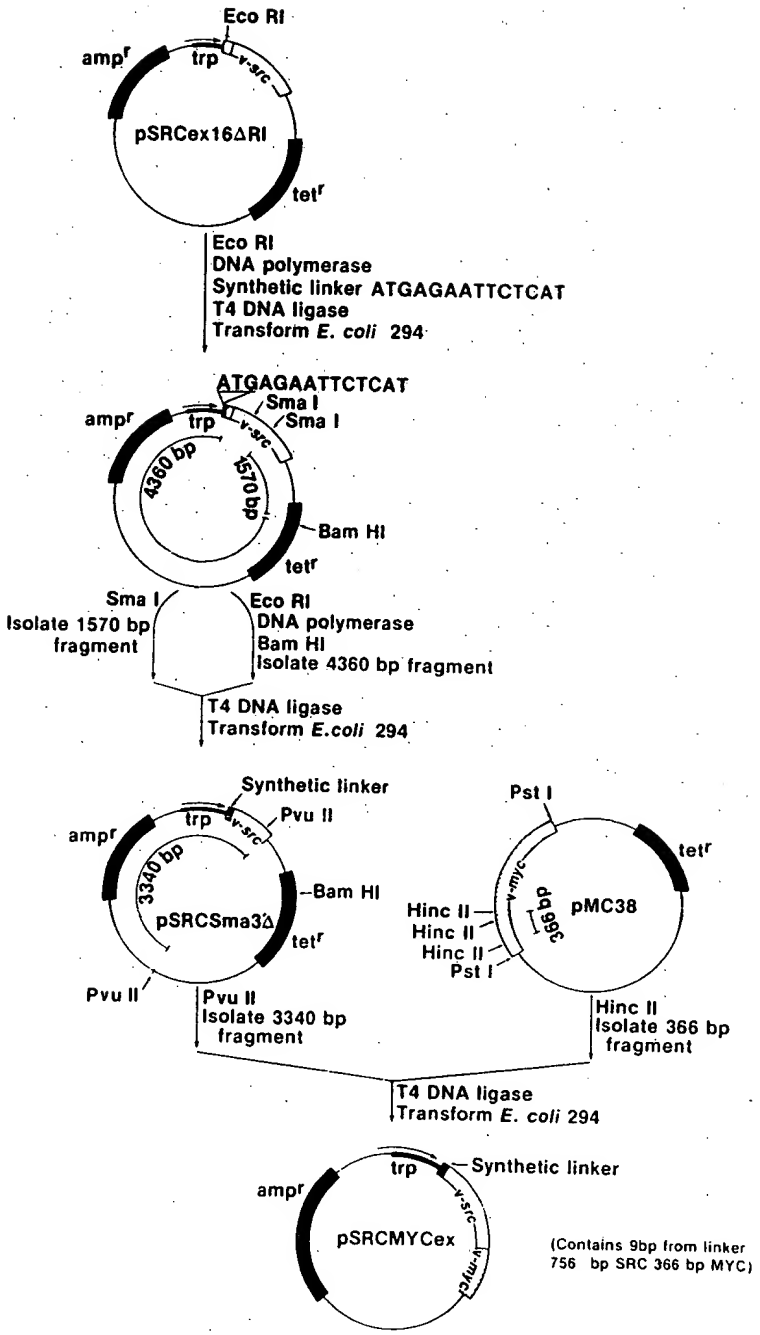

Fig. 9. Construction of a v-*myc* expression vector. A synthetic linker (ATGAGAATTCATCAT) containing a translational initiation codon was inserted downstream from the *trp* promoter in the pSRC ex16 RI expression vector described previously (see ref. 3). Approximately one half of the v-*src* sequences coding for the aminoterminal portion of pp60$^{v\text{-}src}$ protein were then deleted and the remaining portion ligated in translational codon frame with the synthetic ATG. A Hinc II fragment of v-*myc* from plasmid clone MC 38 (nucleotides 320—685 in the v-*myc* sequence in ref. 2) was ligated downstream form remaining v-*src* sequences in continuity with its reading frame. The resulting product contained 3 amino acids from the synthetic linker, 252 amino acids encoded by the 756 base pair fragment from Sma I to Pvu II restriction sites in v-*src* DNA, 122 amino acids from the v-*myc* and 6 amino acids (corresponding to nucleotides 2968—2085) from the pBR322 vector (3).

recent findings on amplified oncogenes, though in many cases the search for an amplified oncogene is still continuing. Even positive findings do not mandate a role for amplified cellular oncogenes, however, because the domain of amplified DNA is inevitably much larger than a single genetic locus (e.g. 38).

ENHANCED EXPRESSION OF AMPLIFIED ONCOGENES

[redacted] (see Table 1). Described cases of elevated RNA expression include examples of abnormal (5, 22) and ectopic (6) transcription. In at least four cases this enhancement is not limited to synthesis of RNA (31, 33, 41, 74, 82). The Y1 cells that have amplified c-Ki-*ras* contain exceptionally large amounts of its protein product situated on the plasma membrane (ref. 74, Fig. 7 and 8). High amounts of the c-*myc* encoded protein are also found in COLO 320 cells that have amplified the gene (33). The *myc* oncogenes have recently been shown to encode nuclear proteins (ref. 1, 3, 26, 29, 32, 33, Fig. 9—11). Both the expression of the c-*myc* mRNA (39) and the subcellular localization of myc proteins are linked to the cell cycle (ref. 89, Fig. 12). It may be that elevated expression of specific c-*myc* functions is necessary for cell cycle progression and the growth transformation aspect of the phenotype of cancer cells that may contribute to tumour progression (7, 36). Elevated expression of c-*myc* has been shown to replace in part platelet-derived growth factor in induction of competence for DNA replication (7). Generally, enhanced expression of an oncogene could be a necessary prerequisite for acquisition of a growth advantage by cells having extra copies of the gene. This effect could also be the principal contribution of amplification to tumourigenesis.

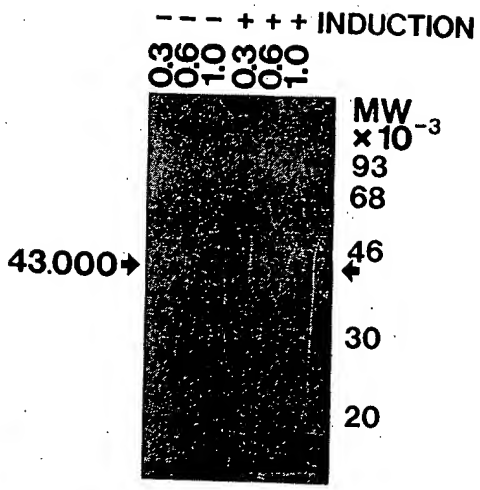

Fig. 10. *E. coli* 294 was transfected with the hybrid v-*src*, v-*myc* plasmid outlined in Fig. 9 and ampicillin-resistant bacterial colonies were checked for the production of a 43,000 m.w. bacterial v-*myc* protein after induction by growth to different optical densities in minimal essential medium (M9, induction+) or complete medium (LB, induction—) (3).

TUMOUR CELL AND STAGE SPECIFICITY OF ONCOGENE ACTIVATION AND AMPLIFICATION

Tumour cell specificity of oncogene amplification has been found in three malignancies. The c-*myc*, L-*myc* or N-*myc* oncogene is amplified in most cases of the variant form of small-cell lung cancer cells (53, 69), c-*erb*B is amplified in several glioblastomas (Josef Schlessinger, personal communication) and the putative N-*myc* oncogene is amplified in about half of grade III and IV neuroblastomas (14, 72, 73, 75). In addition to HSR:s, small-cell lung cancers and neuroblastomas frequently show a

Fig. 11. Indirect immunofluorescence for the v-*myc* protein and phase contrast microscopy of myelocytomatosis virus-transformed quail cells (3). A. Quail cells transformed with the MC-29 virus (Q8 cells). Anti-*myc* protein staining. B. Phase contrast microscopy of field in A. C. Q8 cells stained with anti-*myc* protein antiserum that has been blocked with the immunogen. D. Phase contrast microscopy of field in B. E. Q8 cell stained with preimmune rabbit serum. F. Phase contrast microscopy of field in E.

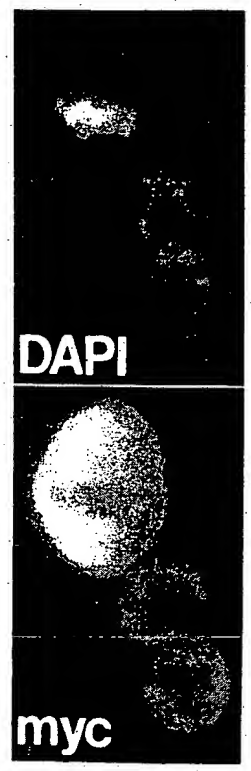

Fig. 12. Fluorescent staining for DNA and *myc* protein in myelocytomatosis virus-transformed quail cells. In interphase cells, the *myc* protein is confined to the nucleae. In the mitotic cell, *myc* fluorescence is distributed throughout the cell unlike fluorescence for chromatin, which is compacted to chromosomes in the metaphase plate. In fact, there is less *myc* fluorescence associated with chromatin than with the rest of the cell. DAPI, diaminophenylindole DNA stain. The anti-*myc* protein rabbit antiserum was used in a 1/200 dilution (ref. 89).

deletion of a portion of the short arm of chromosome 1 (13) and chromosome 3 (86, 87), respectively, in karyological examination. Two kinds of changes have also been described in different neuroblastoma oncogenes. The first is a mutation in the N-*ras* gene, an activated oncogene that was discovered because of its relation to other *ras* genes and transforming activity in transfection experiments (77). The second is amplification of a distant homologue of the c-*myc* gene called N-*myc* (72, 73, 75). Although the transforming potential of the N-*myc* gene has not yet been established, its consistent presence in a core segment of amplified neuroblastoma DNA (38, 57, 72, 73, 75) and its elevated expression in most retinoblastomas (48) suggests its oncogenic nature.

Taya et al. (80) have recently described a human lung giant cell carcinoma grown in nude mice, where both c-Ki-*ras* and c-*myc* oncogenes were amplified about 10-fold. Besides, sequencing studies indicated that at least some of the amplified c-Ki-*ras* copies were also mutationally activated in the 12th codon. These results fit to the multistage theory of cancer development and progression (see 58). Apparently co-operating lesions in cellular oncogenes accumulate during tumour growth and selection and increase the malignant potential of the tumour cells (44).

When does oncogene amplification come into play during tumourigenesis? Gene amplification may not be any initiating event in carcinogenesis. Amplification and enhanced expression of c-*myc* and N-*myc* may occur during the progression of human carcinoma of the lung and neuroblastoma cells to a more malignant phenotype (14, 53, 73). There may be, however, no mandatory sequence of oncogene amplifications for the genesis of any particular tumor. Amplification of an oncogene could play its part in malignant progression of already initiated cells whenever it happened to occur.

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REFERENCES

1. *Abrams HD, Rohrschneider LR, Eisenman RN:* Nuclear location of the putative transforming protein of avian myelocytomatosis virus. Cell 29: 427—439, 1982
2. *Alitalo K, Bishop JM, Smith DH, Chen EY, Colby WW, Lenvinson AD:* Nucleotide sequence of the v-*myc* oncogene of avian retrovirus MC29. Proc Natl Acad Sci USA 80: 100—104, 1983
3. *Alitalo K, Ramsay G, Bishop JM, Ohlsson-Pfeifer S, Colby WW, Levinson AD:* Identification of nuclear proteins encoded by viral and cellular *myc* oncogenes. Nature 306: 274—277, 1983
4. *Alitalo K, Saksela K, Winqvist R, Schwab M, Bishop JM:* Amplification and aberrant expression of cellular oncogenes in human colon cancer cells. In: Genes and cancer. Ed. J. M. Bishop and J. Rowley. Alan Liss Co, New York, in press
5. *Alitalo K, Schwab M, Lin CC, Varmus HE, Bishop JM:* Homogeneously staining chromosomal regions con-

tain amplified copies of an abundantly expressed cellular oncogene (c-*myc*) in malignant neuroendocrine cells from a human colon carcinoma. Proc Natl Acad Sci USA 80: 1707—1711, 1983

6. *Alitalo K, Winqvist R, Lin CC, de la Chapelle A, Schwab M, Bishop JM:* Aberrant expression of an amplified c-*myb* oncogene in two cell lines from a colon carcinoma. Proc Natl Acad Sci USA 81: 4534—4538, 1984
7. *Armelin HA, Armelin MCS, Kelly K, Stewart T, Leder P, Cochran BH, Stiles CD:* Functional role for c-*myc* in mitogenic response to platelet-derived growth factor. Nature 310: 655—660, 1984
8. *Barker PE:* Double Minutes in human tumor cells. Cancer Genet Cytogenet 5: 81—94, 1982
9. *Barker PE, Drwinga HL, Mittelman WN, Maddox AM:* Double minutes replicate once during S phase of the cell cycle. Exp Cell Res 130: 353—360, 1980
10. *Barsoum J, Varshavsky A:* Mitogenic hormones and tumor promoters greatly increase the incidence of colony-forming cells bearing amplified dihydrofolate reductase genes. Proc Natl Acad Sci USA 80: 5330—5334, 1983
11. *Biedler JL, Meyers MB, Spengler BA:* Homogeneously staining regions and double minute chromosomes, prevalent cytogenetic abnormalities of human neuroblastoma cells. Adv Cell Neurobiol 4: 268—301, 1983
12. *Biedler JL, Spengler BA:* Metaphase chromosome anomaly: association with drug resistance and cell-specific products. Science 191: 185—187, 1976
13. *Brodeur GM, Green AA, Hayes FA, Williams KJ, Williams DL, Tsiatis AA:* Cytogenetic features of human neuroblastomas and cell lines. Cancer Res 41: 4678—4686, 1981
14. *Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM:* Amplification of N-*myc* in Untreated Human Neuroblastomas Correlates with Advanced Disease Stage. Science 224: 1121—1124, 1984
15. *Brown PC, Tlsty TD, Schimke RT:* Enhancement of methotrexate resistance and DHFR gene amplification by treatment of 3T6 cells with hydroxyurea. Mol Cell Biol 3: 1097—1107, 1983
16. *Bullock P, Botchan M:* Molecular events in the excicion of SV40 DNA from the chromosomes of cultured mammalian cells. In: Gene amplification. Ed. R. T. Schimke. Cold Spring Harbor Lab 215—230, 1982
17. *Calabretta B, Robberson DL, Barrera-Saldana HA, Lambrou TP, Saunders GF:* Genome instability in a region of human DNA enriched in Alu repeat sequences. Nature 296: 219—225, 1982
18. *Chattopadhyay SK, Chang EH, Lander MR, Ellis RW, Scolnick EM, Lowy DR:* Amplification and rearrangement of *onc* genes in mammalian species. Nature 296: 361—363, 1982
19. *Cifone MA, Fidler IJ:* Increasing metastatic potential is associated with increasing genetic instability of clones isolated from murine neoplasms. Proc Natl Acad Sci USA 78: 6949—6952, 1981
20. *Collins S, Groudine M:* Amplification of endogenous *myc*-related DNA sequences in a human myeloid leukemia cell line. Nature 298: 679—681, 1982
21. *Collins SJ, Groudine MT:* Rearrangement and amplification of c-*abl* sequences in the human chronic myelogenous leukemia cell line K-562. Proc Natl Acad Sci USA 80: 4813—4817, 1983
22. *Collins SJ, Kubonishi I, Miyoshi I, Groudine MT:* Altered transcription of the c-*abl* oncogene in K-562 and other chronic myelogenous leukemia cells. Science 225: 72—74, 1984
23. *Cowell JK:* Double minutes and homogeneously staining regions: Gene amplification in mammalian cells. Ann Rev Genet 16: 21—52, 1982
24. *Cox D, Yuncken C, Spriggs AI:* Numerous minute chromatin bodies in malignant tumours of childhood. Lancet I: 55—58, 1965
25. *Dalla-Favera RD, Wong-Staal F, Gallo RG: Onc* gene amplification in promyelocytic leukemia cell line HL-60 and primary leukaemic cells of the same patient. Nature 299: 61—63, 1982
26. *Donner P, Greiser-Wilke I, Moelling K:* Nuclear Localization and DNA binding of the transforming gene product of avian myelocytomatosis virus. Nature 296: 262—266, 1982
27. *Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD:* Close similarity of epidermal growth factor receptor and v-*erb*B oncogene protein sequences. Nature 307: 521—527, 1984
28. *Echols H:* SOS-functions, cancer and inducible evolution. Cell 25: 1—2, 1981
29. *Eisenman RN, Tachibana CY, Abrams HD, Hann SR:* v-*myc* and c-*myc*-Encoded proteins are associated with the nuclear matrix. Submitted
30. *Gilbert F, Balaban G, Brangman D, Herrmann N, Lister A:* Homogeneously staining regions and tumorigenicity. Int J Cancer 31: 765—768, 1983
31. *Gill GN, Lazar CS:* Increased phosphotyrosine content and inhibition of proliferation in EGF-treated A431 cells. Nature 293: 305—307, 1981
32. *Hann SR, Abrams HD, Rohrschneider LR, Eisenman RN:* Proteins encoded by v-*myc* and c-*myc* oncogenes: Identification and localization in acute leukemia virus transformants and bursal lymphoma cell lines. Cell 34: 789—798, 1983
33. *Hann SR, Eisenman RN:* Two proteins encoded by the human c-*myc* oncogene: Differential expression in neoplastic cells. Mol Cell Biol 4: 2486—2497, 1984
34. *Harper ME, Franchini G, Love J, Simon MI, Gallo RC, Wong-Staal F:* Chromosomal sublocalization of human c-*myb* and c-*fes* cellular onc genes. Nature 304: 169—171, 1983
35. *Heisterkamp N, Stephenson JR, Groffen J, Hansen PF, de Klein A, Bartman CR, Grosveld G:* Localization of the c-*abl* oncogene adjacent to a translocation break point in chronic myelocytic leukaemia. Nature 306: 239—242, 1983
36. *Heldin C-H, Westermark B:* Growth factors: Mechanism of action and relation to oncogenes. Cell 37: 9—20, 1984
37. *Johnston RN, Beverley SM, Schimke RT:* Rapid spontaneous dihydrofolate reductase gene amplification shown by fluorescence-activated cell sorting. Proc Natl Acad Sci USA 80: 3711—3715, 1983
38. *Kanda N, Schreck R, Alt F, Bruns G, Baltimore D, Latt S:* Isolation of amplified DNA sequences from IMR-32 human neuroblastoma cells: Facilitation by fluoroscence-activated flow sorting of metaphase chromosomes. Proc Natl Acad Sci USA 80: 4069—4073, 1983
39. *Kelly K, Cochran BH, Stiles CD, Leder P:* Cell-specific regulation of the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factor. Cell 35: 603—610, 1983
40. *Klein G:* Specific chromosome translocations and the genesis of B-cell derived tumors in mice and men. Cell 32: 311—315, 1983
41. *Konopka JB, Watanabe SM, Witte ON:* An alteration of the human c-*abl* protein in K562 leukemia cells unmasks associated tyrosine kinase activity. Cell 37: 1035—1042, 1984
42. *Kovacs G:* Homogeneously staining regions on marker

chromosomes in malignancy. Int J Cancer 23: 299—301, 1979
43. *Kozbor D, Croce CM:* Amplification of the c-*myc* oncogene in one of five human breast carcinoma cell lines. Cancer Res 44: 438—441, 1984
44. *Land H, Parada LF, Weinberg RA:* Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Science 222: 771—778, 1983
45. *Latt SA:* Sister chromatid exchange formation. Ann Rev Genet 15: 11—15, 1981
46. *Laughlin TJ, Taylor JH:* Initiation of DNA replication in chromosomes of Chinese hamster ovary cells. Chromosoma 75: 19—35, 1979
47. *Lavi S:* Carcinogen-mediated amplification of viral DNA sequences in simian virus 40-transformed Chinese hamster embryo cells. Proc Natl Acad Sci USA 78: 6144—6148, 1981
48. *Lee W-H, Murphree AL, Benedict WF:* Expression and amplification of the N-*myc* gene in primary retinoblastoma. Nature 309: 458—460, 1984
49. *Levan A, Manolov G, Clifford P:* Chromosomes of a human neuroblastoma. A new case with accessory minute chromosomes. J Natl Cancer Inst 41: 1377—1387, 1968
50. *Levan A, Levan G, Mitelman F:* Chromosomes and cancer. Hereditas 86: 15—29, 1977
51. *Lewis JA, Biedler JL, Melera PW:* Gene amplification accompanies low level increases in the activity of dihydrofolate reductase in antifolate-resistant Chinese hamster lung cells containing abnormally banding chromosomes. J Cell Biol 94: 418—424, 1982
52. *Lin CC, Alitalo K, Schwab M, George D, Varmus HE, Bishop JM:* Evolution of karyotypic abnormalities and c-*myc* oncogene amplification in a human colonic carcinoma. Submitted for publication
53. *Little CD, Nau MM, Carney DN, Gazdar AF, Minna JD:* Amplification an expression of the c-*myc* oncogene in human lung cancer cell lines. Nature 306: 194—196, 1983
54. *Lozzio CB, Lozzio BB:* Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood 45: 321—334, 1975
55. *Mariani BD, Schimke RT:* Gene amplification in a single cell cycle in Chinese hamster ovary cells. J Biol Chem 259: 1901—1910, 1984
56. *Mark J:* Double minutes-a chromosomal aberration in Rous sarcomas in mice. Hereditas 57: 1—22, 1967
57. *Montgomery KT, Biedler JL, Spengler BA, Melera PW:* Specific DNA sequence amplification in human neuroblastoma cells. Proc Natl Acad Sci USA 80: 5724—5728, 1983
58. *Nowell RC:* The clonal evolution of tumor cell populations. Science 194: 23—28, 1976
59. *Nowell R, Finan J, Favera RD, Gallo RC, Ar-Rushdi A, Romanczuk G, Selden JR, Emanuel BS, Rovera G, Croce CM:* Association of amplified oncogene c-*myc* with an abnormally banded chromosome 8 in a human leukemia cell line. Nature 306: 494—497, 1983
60. *Pall ML:* Gene amplification model of carcinogenesis. Proc Natl Acad Sci USA 78: 2465—2468, 1981
61. *Pelicci P-G, Lanfrancone L, Brathwaite MD, Wolman SR, Dalla-Favera R:* Amplification of the c-*myb* oncogene in a case of human acute myelogenous leukemia. Science 224: 1117—1121, 1984
62. *Pellegrini S, Dailey L, Basilico C:* Amplification and excision of integrated polyoma DNA sequences require a functional origin of replication. Cell. 36: 943—949, 1984
63. *Quinn LA, Moore GE, Morgan RT, Woods LK:* Cell lines from human colon carcinoma with unusual cell products, double minutes, and homogeneously staining regions. Cancer Res 39: 4914, 1979
64. *Rechavi G, Givol D, Canaani E:* Activation of a cellular oncogene by DNA rearrangement: possible involvement of an IS-like element Nature 300: 607—611, 1982
65. *Roberts JM, Buck LB, Axel R:* A structure for amplified DNA. Cell 33: 53—63, 1983
66. *Rowley JD:* Human oncogene locations and chromosome aberrations. Nature 301: 290, 1983
67. *Rowley JD, Testa JR:* Chromosome abnormalities in malignant hematologic diseases. Adv Cancer Res 36: 103—147, 1982
68. *Rowley JD, Ultmann JE eds:* Chromosomes and Cancer: from molecules to man. Academic Press, New York 1983
69. *Saksela K, Bergh J, Lehto V-P, Nilsson K, Alitalo K:* Amplification of the c-*myc* oncogene is characteristic of a subpopulation of human small cell lung cancer. Cancer Res, in press
70. *Schimke RT:* Gene amplification. Cold Spring Harbor Labor 1982
71. *Schimke RT, Brown PC, Kaufman RJ, McGrogan M, Slate DL:* Chromosomal and extrachromosomal localization of amplified dihydrofolate reductase genes in cultured mammalian cells. Cold Spring Harbor Symp Quant Biol 55: 785—797, 1981
72. *Schwab M, Alitalo K, Klempnauer K-H, Varmus HE, Bishop JM, Gelbert F, Brodeur G, Goldstein M, Trent J:* Amplified DNA with limited homology to *myc* cellular oncogne is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature 305: 245—248, 1983
73. *Schwab M, Alitalo K. Varmus HE, Bishop JM:* Amplification of cellular oncogenes in tumor cells. In: The cancer cell. Cold Spring Harbor Press 2: 215—220, 1984
74. *Schwab M, Alitalo K, Varmus HE, Bishop JM, George D:* A cellular oncogene (c-Ki-*ras*) is amplified, overexpressed, and located within karyotypic abnormalities in mouse adreocortical tumour cells. Nature 303: 497—501, 1983
75. *Schwab M, Varmus HE, Bishop JM, Grezeschik K-H, Naylor SL, Sakaguchi AY, Brodeur G, Trent J:* Chromosome localization in normal human cells and neuroblastomas of a gene related to c-*myc*. Nature 308: 288—291, 1984
76. *Selden JR, Emanuel BS, Wang E, Cannizzaro L, Palumbo A, Erikson J, Nowell PC, Rovera G, Croce CM:* Amplified C_λ and c-*abl* genes are on the same marker chromosome in K562 leukemia cells. Proc Natl Acad Sci USA 80: 7289—7292, 1983
77. *Shimizu K, Goldfarb M, Perucho M, Wigler M:* Isolation and preliminary characterization of the transforming gene of human neuroblastoma cell line. Proc Natl Acad Sci USA 80: 383—387, 1983
78. *Shmookler Reis RJ, Lumpkin CK, McGill JR, Riabowol TK, Goldstein S:* Extrachromosomal circular copies of an 'inter-Alu' unstable sequence in human DNA are amplified during in vitro and in vivo ageing. Nature 201: 394—398, 1983
79. *Stark GR, Wahl GM:* Gene amplification. Ann Rev Biochem 53: 447—491, 1984
80. *Taya Y, Hesogai K, Hirohashi S, Shimosato Y, Tsuchiya R, Tsuchida N, Fushimi M, Sekiya T, Nishimura S:* A novel combination of K-*ras* and *myc* amplification accompanied by point mutational activation of K-*ras* in human lung cancer. EMBO J 3: 2943—2946, 1984
81. *Tlsty TD, Brown PC, Schimke TR:* UV radiation facilitates methotrexate resistance and amplification of the dihydrofolate reductase gene in cultured 3T6 mouse cells. Molec Cell Biol 4: 1050—1056, 1984

82. *Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Libermann TA, Sschlessinger J, Downward J, Mayes ELV, Whittle N, Waterfield MD, Seeburg PH:* Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature 309: 418—425, 1984
83. *Wahl GM, de Saint Vincent BR, DeRose ML:* Effect of chromosomal position on amplification of transfected genes in animal cells. Nature 307: 516—520, 1984
84. *Varshavsky A:* On the possibility of metabolic control of replicon 'misfiring': Relationship to emergence of malignant phenotypes in mammalian cell lineages. Proc Natl Acad Sci USA 78: 3673—3677, 1981
85. *Varshavsky A:* Phorbol ester dramatically increases incidence of methotrexate-resistant mouse cells: Possible mechanisms and relevance to tumor promotion. Cell 25: 561—572, 1981
86. *Whang-Peng J, Bunn PA, Kao-Shan CS, Lee EC, Carney DN, Gazdar A, Minna JD:* A Nonrandom chromosomal abnormality, del 3p (14—23), in human small cell lung cancer (SCLC). Cancer Genet Cytogenet 6: 119—134, 1982
87. *Whang-Peng J, Kao-Shan CS, Lee EC, Bunn PA, Carney DN, Gazdar AF, Portlock C, Minna JD:* Deletion 3p (14—23), Double minute chromosomes, and homogeneously staining regions in human small-cell lung cancer. sCSH Laboratory 1982
88. *Winqvist R, Knuutila S, Leprince D, Stehelin D, Alitalo K:* Mapping of amplified c-*myb* oncogene, sister chromatid exchanges and karyotypic analysis of the COLO 205 colon carcinoma cell line. Cancer Genet Cytogenet. in press
89. *Winqvist R, Saksela K, Alitalo K: myc* proteins are not associated with chromatin in mitotic cells. EMBO J 3: 2947—2950
90. *Woodcock DM, Cooper IA:* Evidence for double replication of chromosomal DNA segments as a general consequence of DNA replication inhibition. Cancer Res 41: 2483—2490, 1981
91. *Zabel BU, Naylor SL, Grezeschik K-H, Sakaguchi AY:* Regional assignment of human protooncogene c-*myb* to 6q21—qter. Somatic Cell Molec Genet 10: 105—108, 1984

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Elevated Epidermal Growth Factor Receptor Gene Copy Number and Expression in a Squamous Carcinoma Cell Line

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Abstract

The human epidermal growth factor (EGF) receptor is known to be homologous to the v-*erb* B oncogene protein of the avian erythroblastosis virus. Overexpression of the EGF receptor gene in A431 epidermoid carcinoma cells is due to gene amplification. In this study, a variety of squamous cell carcinomas were examined and one, SCC-15, contained high levels of the EGF receptor as determined by immunoprecipitation via an EGF receptor-specific polyclonal antibody. Using a cloned EGF receptor complementary DNA as a probe, the level of EGF receptor RNA was found to be elevated four-fold in SCC-15 relative to normal cultured keratinocytes. When the same probe was used to identify EGF receptor gene fragments on a genomic DNA blot, the SCC-15 cell line was shown to possess an EGF receptor gene copy number amplified four to five times. Gene amplification results in the enhancement in the level of the EGF receptor in several carcinomas and could be responsible for the appearance of the transformed phenotype in these cells.

Introduction

The epidermal growth factor (EGF)[1] stimulates growth and elicits a wide variety of rapid and delayed responses by binding to high-affinity cell-surface receptors which are 170-kD glycoproteins (1). Recently, EGF receptor peptides have been sequenced and found to be homologous to the avian erythroblastosis virus *erb* B oncogene product (2), suggesting that the EGF receptor gene is the human c-*erb* B oncogene. A431 epidermoid carcinoma cells possess a very large number of EGF receptors (3), and the EGF receptor gene is amplified ~30-fold (4–6). This amplification is responsible for the overexpression of the EGF receptor protein in these cells (4–6).

A cell culture system has been developed permitting serial cultivation of keratinocytes, whose growth is modulated by EGF (7). Such methods have been used to establish cell lines from squamous cell carcinomas of the oral epithelium (8). Because of the role of EGF in keratinocyte development, we quantified EGF receptor protein and RNA in several squamous cell carcinomas. One cell line, SCC-15, was found to contain high amounts of receptor protein and RNA, and a four- to fivefold amplification of the gene.

Methods

The squamous cell carcinomas established by Rheinwald and Beckett (8) were obtained from, and maintained according to the American Type Culture Collection (Rockville, MD). 1623 was originally designated as SCC-15; 1628 as SCC-25; and 1629 as SCC-9 (8). Normal human esophageal epithelial cells were grown as reported (9). Maintenance of other cell lines was as described elsewhere (10). Proteins were labeled with [^{35}S]methionine and immunoprecipitated as previously described (10). PolyA+ RNA was isolated by guanidine isothiocyanate solubilization and CsCl centrifugation (11), and oligo(dT)-affinity chromatography. RNA (Northern) blotting was performed as described (11, 12). High molecular weight DNA was isolated (4) and analyzed by DNA (Southern) blotting (4, 10, 13). The EGF receptor complementary DNA (cDNA) clone pE7 was constructed and isolated from an A431 cDNA library (11). DNA fragments were ^{32}P-labeled by nick translation.

Results

A large number of cell lines were initially screened for EGF receptor levels by determining their ability to be killed by an EGF-pseudomonas exotoxin conjugate, a technique described previously (10). Several squamous cell carcinomas were found to be relatively sensitive to the EGF-toxin conjugate, including SCC-25, SCC-9, and particularly SCC-15, all derived from the human tongue (8). These three cell lines were labeled with [^{35}S]methionine, and their extracts immunoprecipitated with a goat polyclonal antibody to the EGF receptor, affinity-purified as described (10). When compared with A431 cells, which make very large amounts of the EGF receptor, SCC-25 and SCC-9 make moderate amounts and SCC-15 high amounts of the receptor (Fig. 1, lane *a* vs. *e*, *g*, and *c*). Quantitation of the immunoprecipitation data revealed that SCC-15, SCC-25, and SCC-9 make 41, 15, and 4% of the amount of EGF receptor made by A431 cells, respectively.

Because SCC-15 cells had high levels of receptor, polyA+ RNA was isolated from these cells, electrophoretically fractionated on agarose, and analyzed by RNA (Northern) blotting. A cloned A431 cDNA (pE7) encoding the EGF receptor (11) was ^{32}P-labeled and used as a hybridization probe to visualize EGF receptor RNAs. Fig. 2 *A* shows that SCC-15 contains both the 10- and 5.6-kilobase species of EGF receptor RNA. The levels are approximately four- to fivefold higher than those found in either KB or A498 kidney carcinoma cells; these cell lines were previously found to possess readily de-

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1. *Abbreviations used in this paper:* cDNA, complementary DNA; EGF, epidermal growth factor.

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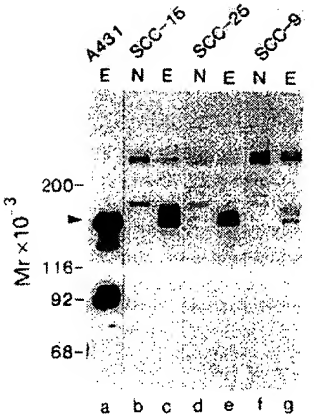

Figure 1. Autoradiograph of electrophoresed ^{35}S-labeled EGF receptor protein (arrow) immunoprecipitated by either E, affinity-purified goat polyclonal EGF receptor antibody, or N, normal serum. Molecular weight markers are at left.

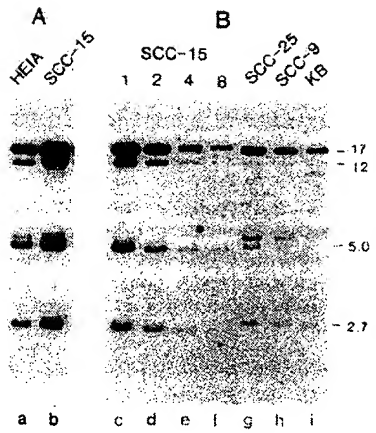

Figure 3. DNA blot analysis of *Hin*dIII-digested genomic DNAs using the pE7 probe (see Fig. 2). (*A*) and (*B*) are autoradiographs from two separate gels. Sizes are in kilobase pairs (right). 10 μg of DNA was loaded except in (*B*), lanes *d–f*, which represent serial dilutions of the 1623 DNA shown in lane *c*.

tectable levels of both receptor RNAs (10). Fig. 2 *B* shows that cultured human epithelial cells contain EGF receptor-specific RNA (HEIA, lane *e*) whose levels are higher than an early passage human fibroblast D551 (lane *f*), equivalent to A498 (lane *g*), but much lower than SCC-15 (lane *b*).

To determine if an elevated gene copy number was associated with enhanced expression of the EGF receptor gene in SCC-15 cells, genomic DNA was isolated from normal cultured epithelial cells (HEIA) and SCC-15 cells, digested with *Hin*dIII, electrophoretically fractionated, and subjected to DNA blotting analysis. An EGF receptor cDNA (pE7) was used as a hybridization probe to identify receptor DNA fragments. Fig. 3 *A* reveals that the SCC-15 genome contains four- to fivefold amplified EGF receptor gene sequences relative to normal epithelial cells (lane *a* vs. *b*). Analysis of β-actin gene fragments on the same filter by hybridization to a chick actin cDNA probe indicated that equal amounts of DNA were loaded per well (data not shown). Digested SCC-15 DNA had to be diluted about fourfold (Fig. 3 *B*, lane *e*) to approximate the signal intensity of receptor DNA fragments from SCC-25, SCC-9, and KB cells (lanes *g–i*). The KB cell EGF receptor gene is known not to be amplified (10).

Discussion

It may be significant that A431 carcinoma cells are not unique in their possession of amplified EGF receptor genes. We report here that the EGF receptor gene in squamous cell carcinoma SCC-15 is amplified four- to fivefold relative to normal epithelial cells. The amplification of the EGF receptor gene may cause the initiation or maintenance of the malignant state in some human cells.

Previously, we reported that a variety of transformed cell lines synthesize relatively high amounts of both EGF receptor protein and messenger RNA (10). It is conceivable that a moderate or even a small increase in the level of the EGF receptor leads to a change in the cellular phenotype, as has been demonstrated for the *src* gene product (14). If this hypothesis is correct, then even a minor amplification of the EGF receptor gene copy number could contribute to the onset of tumorigenesis. Hendler and Ozanne (15) have examined lung squamous cell carcinomas and found that they contain a 2.5–5-fold increase in EGF receptor levels.

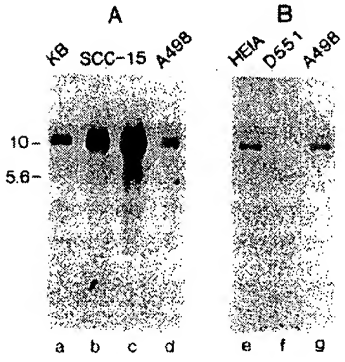

Figure 2. RNA blot analysis of polyA+ RNAs using the ^{32}P-labeled EGF receptor cDNA probe pE7. (*A*) and (*B*) are autoradiographs from two separate gels. Sizes are in kilobases (left). 5 (lanes *a*, *b*, *d*, and *g*) or 10 (lanes *c*, *e*, and *f*) μg of RNA were loaded.

References

1. Savage, C. R., and S. J. Cohen. 1972. Epidermal growth factor and a new derivation: rapid isolation procedure and biological and chemical characterization. *J. Biol. Chem.* 247:7609–7611.

2. Downward, J., Y. Yarden, E. Mayes, G. Scarce, N. Totty, P. Stockwell, A. Ullrich, J. Schlessinger, and M. D. Waterfield. 1984. Close similarity of the epidermal growth factor receptor and v-erb B oncogene protein sequence. *Nature (Lond.).* 307:521–527.

3. Fabricant, R. N., J. E. DeLarco, and G. J. Todaro. 1977. Nerve growth factor receptor on human melanoma cells in culture. *Proc. Natl. Acad. Sci. USA.* 74:565–569.

4. Merlino, G. T., Y.-H. Xu, S. Ishii, A. J. L. Clark, K. Semba, K. Toyoshima, T. Yamamoto, and I. Pastan. 1984. Amplification and enhanced expression of the epidermal growth factor receptor gene in A431 human carcinoma cells. *Science (Wash. DC).* 224:417–419.

5. Ullrich, A., L. Coussens, J. S. Hayflick, T. J. Dull, A. Gray, A. W. Tam, J. Lee, Y. Yarden, T. A. Libermann, J. Schlessinger, J. Downward, E. L. V. Mayes, N. Whittle, M. D. Waterfield, and P. H. Seeburg. 1984. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermal carcinoma cells. *Nature (Lond.).* 309:418–425.

6. Lin, C. R., W. S. Chen, W. Kruiger, L. S. Stolarsky, W. Weber, R. M. Evans, I. M. Verma, G. N. Gill, and M. G. Rosenfeld. 1984.

Expression cloning of human EGF receptor complementary DNA: gene amplification and three related messenger RNA products in A431 cells. *Science (Wash. DC).* 224:843–848.

7. Rheinwald, J. G., and H. Green. 1975. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell.* 6:331–344.

8. Rheinwald, J. G., and M. A. Beckett. 1981. Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultured from human squamous cell carcinomas. *Cancer Res.* 41:1657–1663.

9. Banks-Schlegel, S. P., and C. C. Harris. 1983. Tissue-specific expression of keratin proteins in human esophageal and epidermal epithelium and their cultured keratinocytes. *Exp. Cell Res.* 146:271–280.

10. Xu, Y.-H., N. Richert, S. Ito, G. T. Merlino, and I. Pastan. 1984. Characterization of epidermal growth factor receptor gene expression in malignant and normal human cell lines. *Proc. Natl. Acad. Sci. USA.* 81:7308–7312.

11. Xu, Y.-H., I. Ishii, A. J. L. Clark, M. Sullivan, R. K. Wilson, D. P. Ma, B. A. Roe, G. T. Merlino, and I. Pastan. 1984. Human epidermal growth factor receptor cDNA is homologous to a variety of RNAs overproduced in A431 carcinoma cells. *Nature (Lond.).* 309: 806–810.

12. Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA.* 77:5201–5205.

13. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503–517.

14. Jakobovits, E. B., J. E. Majors, and H. E. Varmus. 1984. Hormonal regulation of the Rous sarcoma src gene via a heterologous promoter defines a threshold dose for cellular transformation. *Cell.* 38: 757–765.

15. Hendler, F. J., and B. W. Ozanne. 1984. Human squamous cell lung cancers express increased epidermal growth factor receptors. *J. Clin. Invest.* 74:647–651.

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Cyclin A and cyclin D1 as significant prognostic markers in colorectal cancer patients

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Abstract

Background: Colorectal cancer is a common cancer all over the world. Aberrations in the cell cycle checkpoints have been shown to be of prognostic significance in colorectal cancer.

Methods: The expression of *cyclin D1*, *cyclin A*, *histone H3* and *Ki-67* was examined in 60 colorectal cancer cases for co-regulation and impact on overall survival using immunohistochemistry, southern blot and in situ hybridization techniques. Immunoreactivity was evaluated semi quantitatively by determining the staining index of the studied proteins.

Results: There was a significant correlation between *cyclin D1* gene amplification and protein overexpression (concordance = 63.6%) and between *Ki-67* and the other studied proteins. The staining index for *Ki-67, cyclin A and D1* was higher in large, poorly differentiated tumors. The staining index of *cyclin D1* was significantly higher in cases with deeply invasive tumors and nodal metastasis. Overexpression of *cyclin A* and *D1* and amplification of *cyclin D1* were associated with reduced overall survival. Multivariate analysis shows that *cyclin D1 and A* are two independent prognostic factors in colorectal cancer patients.

Conclusions: Loss of cell cycle checkpoints control is common in colorectal cancer. *Cyclin A and D1* are superior independent indicators of poor prognosis in colorectal cancer patients. Therefore, they may help in predicting the clinical outcome of those patients on an individual basis and could be considered important therapeutic targets.

Background

Colorectal cancer (CRC) is the third most common cancer in Western countries [1]. In Egypt, CRC has unique characteristics that differ from that reported in other countries of the western society. It was estimated that 35.6% of the Egyptian CRC cases are below 40 years of age and patients

usually present with advanced stage, high grade tumors that carry more mutations [2]. This uniquely high proportion of early-onset CRC, the early and continuous exposure to hazardous environmental agents, the different mutational spectrum and the prevalent consanguinity in Egypt justify further studies [3]. It was proved that most cancers result from accumulation of genetic alterations involving certain groups of genes, the majority of which are cell cycle regulators that either stimulate or inhibit cell cycle progression [1]. Cell proliferation allows orderly progression through the cell cycle, which is governed by a number of proteins including *cyclins* and *cyclin* dependent kinases [4,5]. The *cyclins* belong to a superfamily of genes whose products complex with various *cyclin*-dependent kinases (*cdks*) to regulate transitions through key checkpoints of the cell cycle [6]. Abnormalities of several *cyclins* have been reported in different tumor types, implicating, in particular, *cyclin A*, *cyclin E* and *cyclin D* [6,7].

Cyclin D1 is a G1 *cyclin* that regulates the transition from G1 to S phase since its peak level and maximum activity are reached during the G1 phase of the cell cycle. Whereas *cyclin A* is regarded a regulator of the transition to mitosis since it reaches its maximum level during the S and G2 phases [8]. The mechanisms likely to activate the oncogenic properties of the *cyclins* include chromosomal translocations, gene amplification and aberrant protein overexpression [7,9].

Several studies have shown that, *histone H3* mRNA expression can be used to identify the S phase fraction (SPF) through the in situ hybridization (ISH) technique [10,11]. The level of *histone H3* mRNA reaches its peak during the S phase and then drops rapidly at the G2 phase [12].

In face of the increasing incidence of CRC and its peculiar pattern in the Egyptian population, the present study was conducted to assess the role of *Ki-67* (pan-cell cycle marker), *cyclin D1* (G1 phase marker), *histone H3* mRNA (S phase marker), *cyclin A* (S to G2 phase marker) in CRC. The expression level of these markers was correlated to the clinicopathologic features and the overall survival of patients.

Table 1: Clinicopathological features of patients in relation to the staining index (SI) of *Ki-67*, *cyclin D1*, *cyclin A*, *histone H3*

		SI (mean + SD)			
Variables	*No. of cases*	*Ki-67*	*Cyclin D1*	*Cyclin A*	*Histone H3*
Sex					
Male	36	18.0 ± 6.4	6.7 ± 4.3	12.7 ± 5.7	10.7 ± 5.3
Female	24	20.1 ± 5.8	8.8 ± 8.4	10.0 ± 6.0	10.7 ± 5.4
Age (years)					
≥50	41	11.7 ± 6.0*	5.6 ± 5.2	10.0 ± 5.3	6.0 ± 5.0*
<50	19	23.8 ± 5.6	7.7 ± 6.8	13.6 ± 5.7	22.0 ± 5.2
Tumor size (cm)					
<5.0	33	12.2 ± 6.3*	5.3 ± 3.8*	11.5 ± 6.1*	10.3 ± 4.9*
≥5.0	27	30.1 ± 6.2	22.8 ± 7.2	28.6 ± 5.6	24.0 ± 5.6
Histology					
Normal	20	3.5 ± 2.0*	0.6 ± 0.2*	2.3 ± 1.1*	2.2 ± 0.9
Carcinoma	60	30.3 ± 6.2	24.9 ± 6.3	27.2 ± 5.8	10.7 ± 5.3
GI	15	11.7 ± 6.2	6.6 ± 4.0	10.0 ± 5.4	11.4 ± 4.9
GII	21	11.8 ± 5.6	8.9 ± 3.6	12.3 ± 6.5	7.8 ± 5.4
GIII	24	30.0 ± 4.3	22.0 ± 8.1	27.0 ± 4.9	11.5 ± 5.4
Lymph node					
Negative	33	19.5 ± 7.0	5.4 ± 5.3*	11.9 ± 6.5	12.3 ± 5.5
Positive	27	21.3 ± 4.9	20.6 ± 6.9	12.5 ± 5.0	14.2 ± 5.0
Depth of invasion					
m, sm	17	20.7 ± 6.7	3.1 ± 3.1*	11.9 ± 7.2	10.4 ± 5.1
beyond sm	43	21.9 ± 6.2	12.4 ± 6.5	12.2 ± 5.6	10.7 ± 5.4
Stage					
I	6	20.6 ± 6.7	5.7 ± 6.9	24.2 ± 6.9	11.1 ± 5.3
II	27	20.8 ± 6.9	5.3 ± 4.3	24.6 ± 6.0	10.4 ± 5.7
III	12	22.0 ± 5.4	7.7 ± 6.0	27.1 ± 5.2	10.4 ± 4.9
IV	15	24.7 ± 6.1	11.3 ± 9.6	27.5 ± 5.5	12.3 ± 6.2

* p. value < 0.05 (significant)

Methods

Tissue samples

Paraffin-embedded tumor tissues were obtained from 60 CRC patients (47 colon and 13 rectal carcinomas) that were diagnosed and treated at the National Cancer Institute, Cairo, Egypt during the period from January, 1997 to June, 2002. Clinicopathological data of the studied cases are illustrated in table 1. None of the patients received any chemotherapy or irradiation prior to surgery. Histological diagnosis of all cases was done by 2 independent pathologists according to the WHO Histological Classification. Tumors were staged according to the TNM staging system [13]. The depth of tumor invasion was classified as invasion of the mucosa including muscularis mucosa (m), invasion of the submucosa (sm), or invasion beyond the submucosa [8]. Normal colonic tissues were obtained from autopsy specimens (n = 20) and were used as a control. The actual survival rate of the patients was calculated from the date of resection to the date of death.

Immunohistochemistry

Four micron sections of each normal and tumor specimen were cut onto positive-charged slides; air dried overnight, de-paraffinized in xylene, hydrated through a series of graded alcohol and washed in distilled water and 0.01 PBS (pH 7.4). Slides were then processed for IHC as described by Handa et al. [8]. using the following antibodies: *Ki-67* (MIB-1, Dako), *cyclin A* (6E6; Novocastra, Newcastle-Upon-Tyne, UK) and *cyclin D1* (DCS-6, Dako). A case of invasive breast cancer was used as a positive control for *Ki-67* and *cyclin A* whereas a case of mantle cell lymphoma was used as a control for *cyclin D1*. Negative controls were obtained by replacing the primary antibody by non-immunized rabbit or mouse serum.

Brown nuclear staining was regarded as a positive result for all studied markers. The proportion of positively-stained cells and the intensity of staining were scored in tumor and normal colorectal mucosal sections at medium power (×200). The degree of positive tumor staining (percentage of positive tumor cells in the examined section) was scored from 1–6 and the staining intensity was scored from 0–6 according to the pattern of staining in the examined section. Staining index (SI) was calculated by multiplying the cellularity and staining scores as described by King et al. [14].

In situ hybridization

All tumor samples and 5 normal controls were assessed for *histone H3* mRNA by ISH using the commercially available 550 base fluorescein-labeled DNA probe (Dako, Carpinteria, CA) as described by Nagao et al., 1996. This probe hybridizes to the whole mRNA transcript of the human *histoneH3* gene including the5' and 3' un-translated regions. Scoring of *histone H3* mRNA was performed as for immunohistochemistry, however, hybridization signals were detected in the cytoplasm.

Molecular detection of cyclin D1 gene amplification

High molecular weight DNA was extracted from paraffin-embedded tissues of the tumor and normal colorectal mucosal samples as previously described [15]. The proportion of neoplastic and normal cells was determined in each tumor sample by examining hematoxylin and eosin-stained slides obtained from the edge of the specimen used for DNA extraction. Tumor samples were evaluated for amplification of *cyclin D1* if more than 75% of the examined sections were formed of neoplastic cells. Accordingly, 50 cases were eligible for the analysis. Ten micrograms of the extracted DNA was digested with *Eco*R1. DNA from selected cases was also digested with *Bgl*II and *Hind*III. Samples were separated on 0.8% agarose gels and transferred to Hybond-N membranes (Amersham Int., Amersham, UK). The membranes were hybridized with 50% formamide, 5 × SSC, 5 × Denhardt's, 500 μg/ml denatured salmon sperm DNA, 10% dextran sulphate and 10^6 cpm/ml of ^{32}P-labeled *PRAD-1* probe for 24 h. Membranes were washed with 2 × SSC, 0.1% SDS at room temperature for 30 min followed by 2 × SSC, 0.1% SDS at 60°C for 30 min and 0.1 × SSC, 0.1% SDS at 60°C for 1 h. Filters were autoradiographed using an intensifying screen at -70°C for 24–72 h. After being stripped free of the *PRAD-1* probe, the same blots were hybridized with ^{32}P-labeled *B-actin* probe to normalize against possible variations in the loading or transfer of DNA. The autoradiograms were analyzed using a densitometer. Intensities of *PRAD-1/cyclin D1* were normalized to the *β-actin* control bands. The degree of amplification was calculated from these normalized values. Amplification was considered when the signal of the tumor band was ≥2-fold the value of the matched normal mucosa [16].

Statistical analysis

The Mann-Whitney non-parametric test was used to compare the SIs of pairs of subjects whereas the Kruskal-wallis was used for categorial data. Correlation between indices was performed using a simple linear regression test. The Kaplan-Meier method was used to create survival curves which were analyzed by the log-rank test. The impact of different variables on survival was determined using the Cox proportional hazards model. *p*. values less than 0.05 were considered significant.

Results

The results of IHC are illustrated in figures 1 and 2. In general, the staining index (SIs) of all studied markers was higher in carcinomas than in normal colonic mucosal samples ($p = 0.0001$). Normal colorectal mucosa revealed positive imunostaining for *Ki-67* in the lower half of the crypts only. A heterogeneous staining pattern was

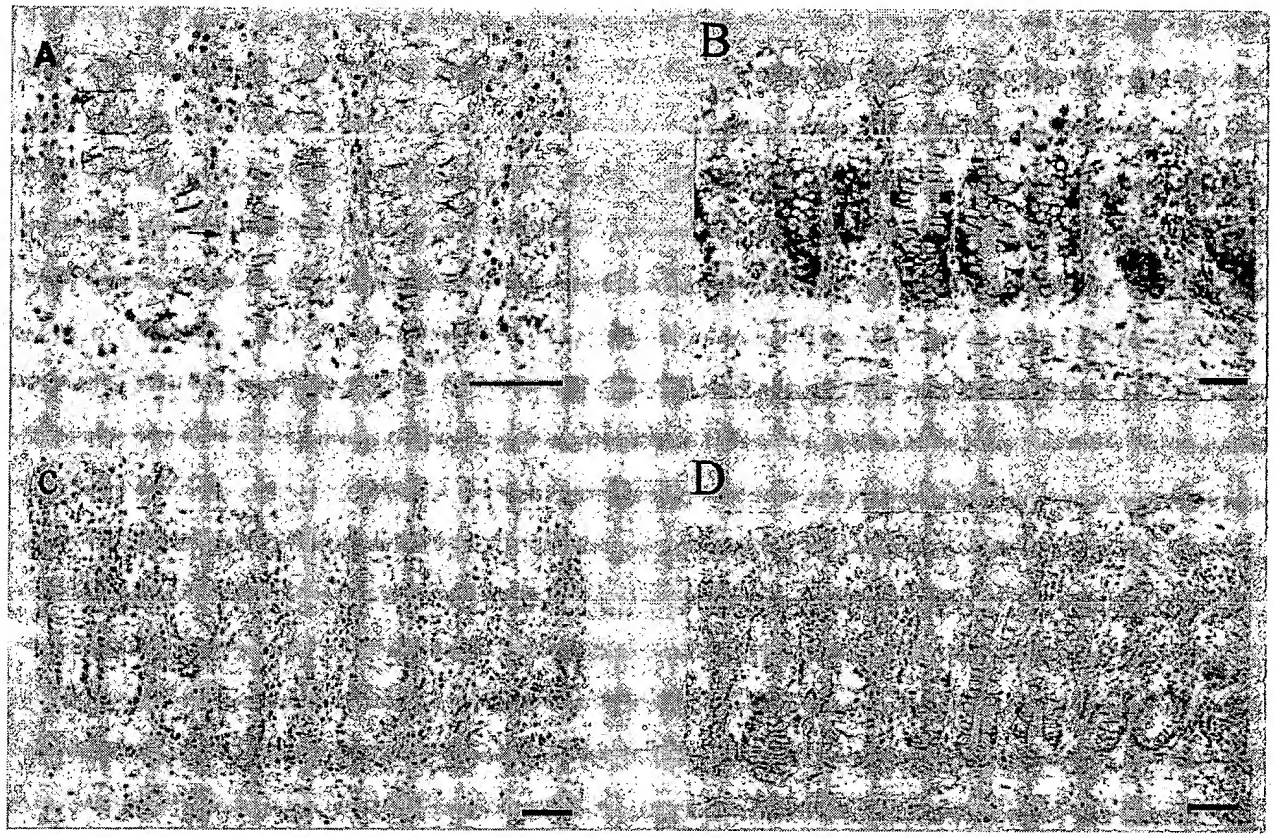

Figure 1
Normal colonic mucosa showing positive nuclear immunostaining for: (a) *cyclin D1*, (b) ISH of *histone H3* mRNA, (c) *Ki-67* and (d) *cyclin A*

detected in the neoplastic cells of well and moderately-differentiated adenocarcinomas whereas a diffuse homogeneous staining pattern was detected in poorly-differentiated carcinomas. The SI ranged from 10–40.2 (mean: 24.6 ± 6.5).

Immunostaining for *cyclin D1* was predominantly nuclear but cytoplasmic staining was detected in some cases. However, unless a nuclear staining was also detected, cases with cytoplasmic staining were considered negative. Normal colorectal mucosal samples were almost negative for *cyclin D1* whereas 41 out of the 60 (68.3%) CRC cases were positive. Marked heterogeneity was observed in well- and moderately-differentiated adenocarcinomas even within the same tumor. Poorly-differentiated carcinomas revealed a diffuse staining pattern with more darkly-stained nuclei. The SI ranged from 0.5–28.6 (mean: 9.3 ± 4.2).

Positive nuclear staining for *cyclin A* was detected in 80% (48/60) of CRC cases and in all non-neoplastic control samples. Positively-stained nuclei were confined to the lower half of the crypts in normal colonic mucosa and diffusely-dispersed in carcinomas. The SI ranged from 3.3–30.2 (mean: 15.1 ± 6.6).

Histone H3 mRNA was intensely expressed in the cytoplasm of all examined samples either neoplastic or non-neoplastic. The distribution of *histone H3* mRNA was similar to that of *cyclin A* and *Ki-67* however, the proportion of *histone H3* mRNA positive cells was less than that of *Ki-67*. The SI ranged from 1.8–24.2 (mean: 12.4 ± 5.3).

The *PRAD-1* probe detected 3 *Eco*RI fragments of 4.0, 2.2 and 2.0 and 1 *Bgl*II fragment of 15 Kb. *PRAD-1/cyclin D1* gene amplification was detected in 22/50 (44%) cases analyzed. The degree of amplification was heterogeneous

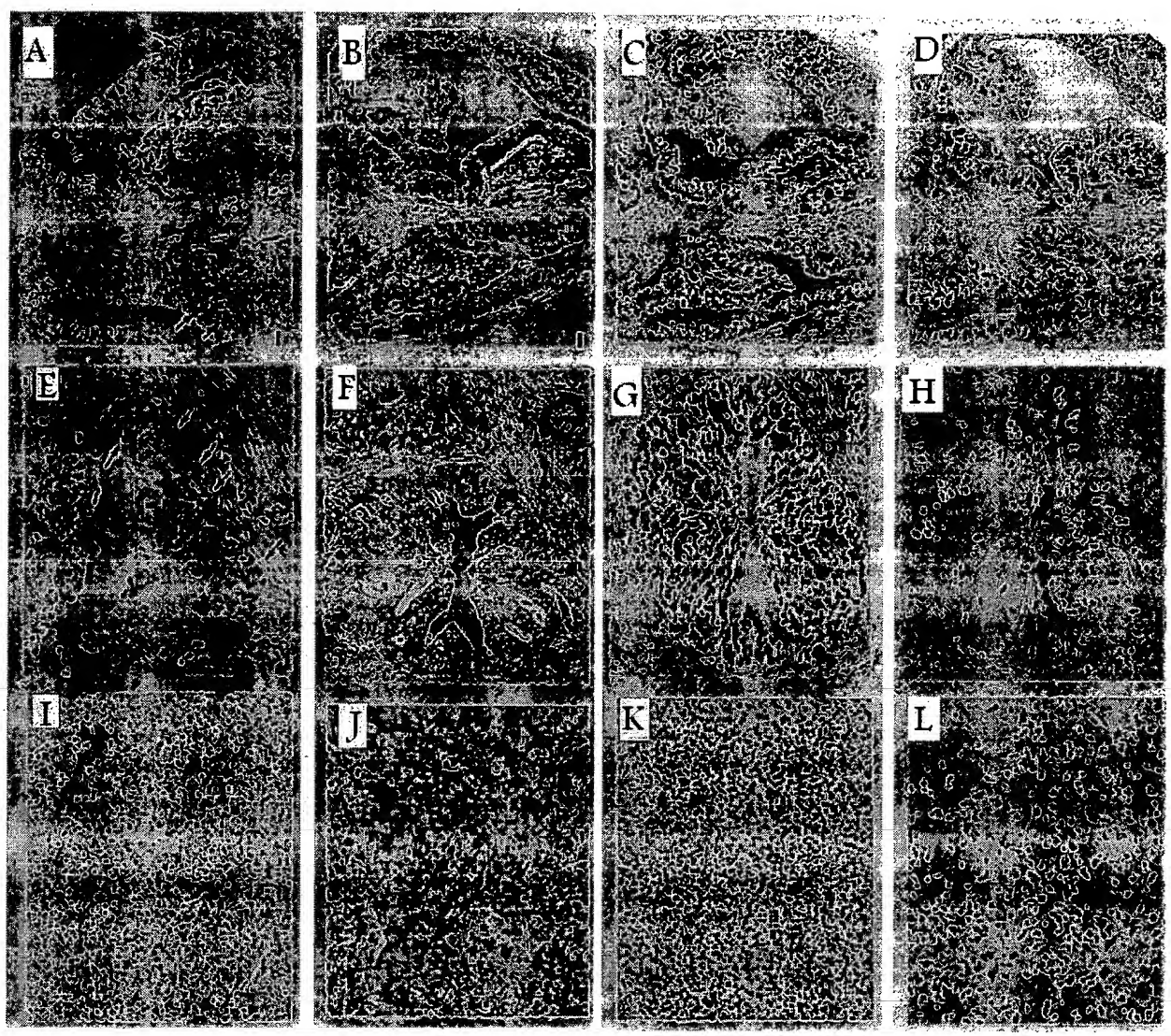

Figure 2
A case of well differentiated adenocarcinoma with positive immunostaining for: (a) *cyclin D1*, (b) *histone H3* mRNA, (c) *Ki-67*, and (d) *cyclin A*. Another case of moderately differentiated denocarcinoma with positive immunostaining for: (e) cyclin D1, (f) *histone H3* mRNA, (g) *Ki-67*, and (h) *cyclin A*. A case of poorly differentiated adenocarcinoma with diffuse staining for: (i) *cyclin D1*, (j) ISH of *histone H3* mRNA, (k) *Ki-67* and (l) *cyclin A*.

with 2–10 fold increase when compared to normal mucosal samples (Figure 3). Amplification was confirmed by other restriction enzymes.

Correlations

There was a significant correlation between *cyclin D1* gene amplification and protein overexpression. Out of the 22 cases that showed amplification 14 showed protein overexpression (concordance = 63.6%).

Linear regression analysis of SIs revealed a significant correlation between *Ki-67* and *cyclin D1*, *cyclin A*, *histone H3* as well as between the SIs of *cyclin A* and *histone H3* (p = 0.008, 0.0001, and 0.0001 respectively) (Figure 4). There was a significant relationship between the SI of both *Ki-67*

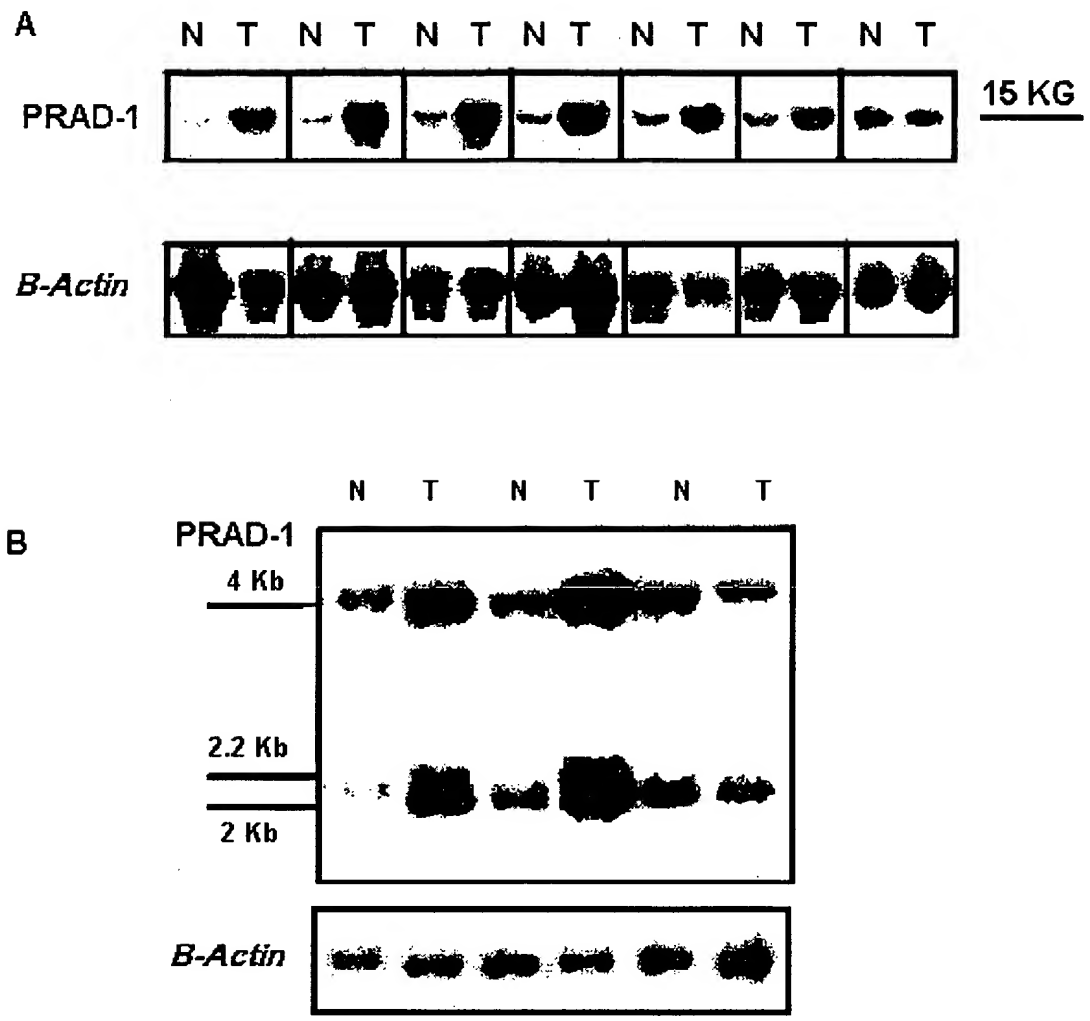

Figure 3

A: Southern blot analysis of normal mucosa (N) and their seven corresponding cases of colonic adenocarcinomas (T1–T7), cases No. 1, 2, 4, and 5 are poorly differentiated whereas cases No. 3, 6, and 7 are moderately differentiated. Genomic DNA was digested with *Bgl*II, fractionated by electrophoresis in agarose gel, transferred onto membranes and hybridized with *PRAD1* and *β-actin*. Tumors number 1–6 (Lanes 1–6) show different degrees of *PRAD1/cyclin D1* amplification, tumor number 7 (lane 7) was not amplified. **B:** Southern blot analysis of 3 cases of adenocarcinomas (T) and matched normal colonic mucosa (N). Genomic DNA was digested with *Eco*RI, fractionated by electrophoresis in agarose gel, transferred onto membranes and hybridized with *PRAD1* and *β-actin* probes for loading control. The identification of the 3 tumors is the same as in Fig. 3A with amplification of *PRAD1/cyclin D1* in tumors number 4, 5 (Lanes 1, 2) but not 7 (Lane 3).

and *cyclin A* and the degree of differentiation of tumors as well as the size of the tumor ($p < 0.001$ and $p < 0.01$ respectively). In addition, SI of *Ki-67* and *histone H3* were higher in patients <50 years than in those ≥50 years ($p < 0.05$) (table 1).

In addition table 2 shows a significant relationship between high *cyclin D1* SI and large, poorly-differentiated tumors, carcinomas with positive lymph node metastasis and deeply-invasive carcinomas ($p < 0.05$, $p < 0.001$, $p < 0.05$ and $p < 0.05$ respectively). Whereas *cyclin D1* gene amplification was significantly associated with an advanced disease stage since amplification was detected in

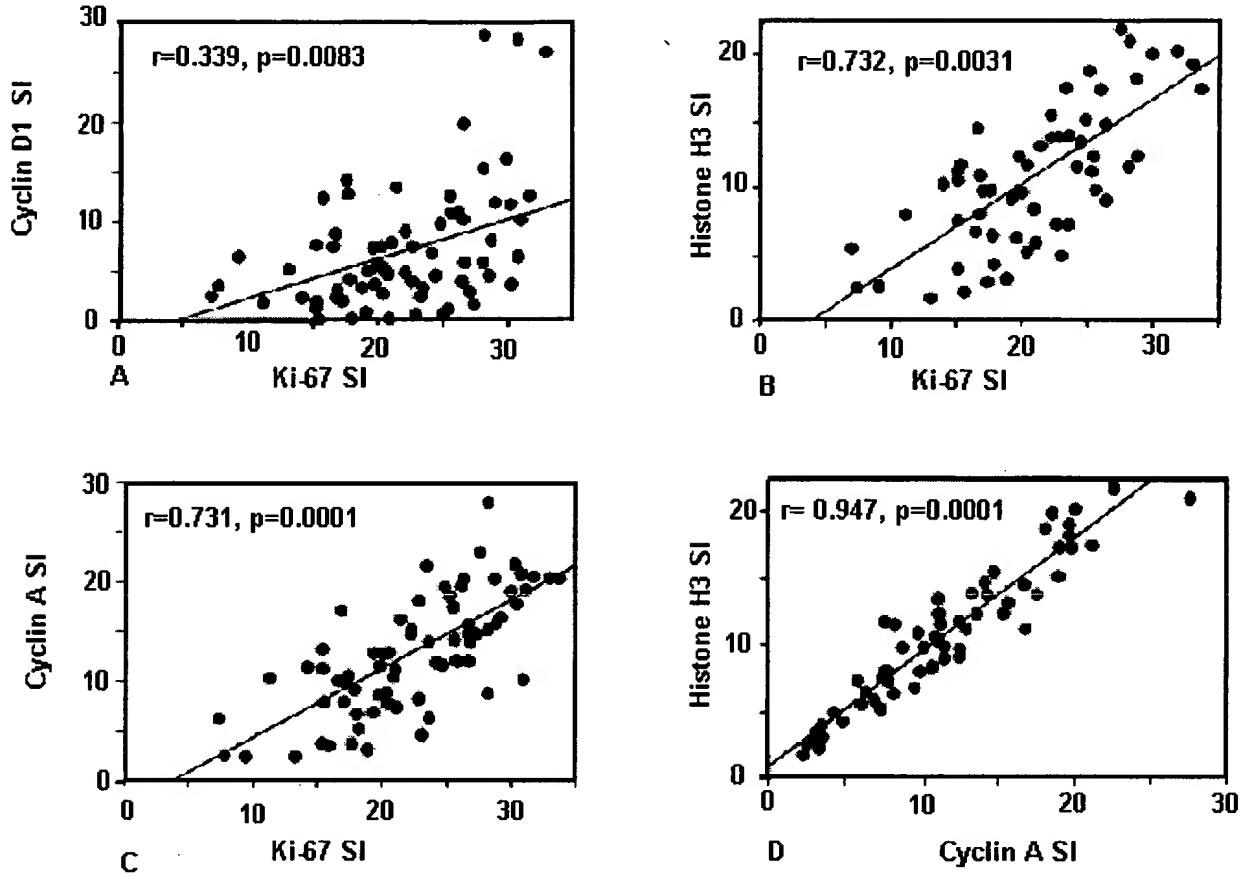

Figure 4
Correlation between the staining intensity of (a) *Ki-67 vs. cyclin D1*, (b) *Ki-67 vs. histone H3*, (c) *Ki-67 vs. cyclin A* and (d) *cyclin A vs. histone H3* mRNA expression.

10/15 (66.7%) of stage IV tumors compared to 12/45 (26.7%) of stage I-III tumors ($p = 0.002$). Similarly, DNA amplification was detected in 60.5% (26/43) of the carcinomas with extensive local invasion (beyond sm) but only in 23.5% (4/17) of the carcinomas with limited invasion (m, sm) ($p = 0.001$). A significant correlation was also present between *cyclin D1* gene amplification and the presence of lymph node metastasis ($p = 0.008$) as well as between the SI of *histone H3*, the size of the tumor and the patient's age ($p < 0.05$, $p < 0.001$ respectively). The SI was higher in tumors >5 cm in diameter and in patients <50 years.

Survival analysis

The mean follow-up period for all patients was 30 months (range: 1-66 months). Eighteen of 60 patients had already died by the time the study was completed. We defined the cutoff level for overexpression of each cell cycle marker at the point that showed the maximum difference of survival rate between the 2 groups separated by that point. Cox regression analysis revealed that *cyclin A* overexpression (our definition: SI ≥ 10.5), *cyclin D1* overexpression (our definition: SI ≥ 6.1), poorly differentiated histology, lymph node metastasis, TNM stage, tumor size and depth of invasion were all significant prognostic variables for survival (Table 3). The Kaplan-Meier survival curves for the subgroups of patients who are subdivided according to each marker's status are shown in Figure 5. Patient with tumors that showed *Ki-67* overexpression (our definition: SI ≥ 11.5) and *histone H3* overexpression (our definition: SI ≥ 8.2) tended to have poor prognosis but this did not reach a statistically significant level, however the overall survival was significantly lower in patient with *cyclin A* and *cyclin D1* overexpression. Cox multivari-

Table 2: The relation between *cyclin D1* overexpression vs *cyclin D1* amplification and clinicopathological prognostic markers.

Variables	*No. of cases*	*Cyclin D1 overexpression*	*Cyclin D1 Amplification*
Tumor size (cm)			
<5.0	33	5.3 ± 3.8*	13/33
≥5.0	27	22.8 ± 7.2 p *<0.05*	9/27 p *<0.236*
Histology			
GI	15	6.6 ± 4.0	7/15
GII	21	8.9 ± 3.6	8/21
GIII	24	22.0 ± 8.1 p *<0.001*	7/24 p *<0.075*
Lymph node			
Negative	33	5.4 ± 5.3*	6/33 (18.2%)
Positive	27	20.6 ± 6.9 p *<0.05*	16/27 (59.3%) p *<0.008*
Depth of invasion			
m, sm	17	3.1 ± 3.1*	4/17 (23.5%)
beyond sm	43	12.4 ± 6.5 p *<0.05*	26/43 (60.5%) p *<0.001*
Stage			
early	45	5.5 ± 10.1	12/45 (26.7%)
late	15	11.3 ± 9.6 P = *0.175*	10/15 (66.7%) p *<0.002*

Table 3: Uunivariate analysis of the relationship between survival and the tested markers

PredictiveVariables	*Median Survival*	*HR*	*CI*	*P*
Ki-67				
<11.5	36			
≥11.5	32	1.826	0.636 – 5.243	0.26
Cyclin D1				
<6.1	35			
≥6.1	18	7.246	1.007 – 45.150	0.03*
Histone H3				
<8.2	35			
≥8.2	29	4.639	0.854 – 25.196	0.07
Cyclin A				
<10.5	35			
≥10.5	15	7.820	1.017 – 60.122	0.02*
Histological grade				
Low	38			
High	10	7.331	2.696 – 19.940	0.0001*
Lymph node				
Negative	38			
Positive	15	6.826	1.973 – 23.621	0.002*
Stage				
I, II, III	38			
IV	12	6.378	1.842 – 22.083	0.001*
Tumor size (cm)				
<5.0	35			
≥5.0	13	4.835	1.386 – 16.868	0.01*
Depth of invasion				
T1, T2	36			
T3, T4	20	7.759	1.024 – 58.789	0.04*
Age (years)				
<50	38			
≥50	28	2.802	0.988 – 7.943	0.0526
Sex				
Male	38			
Female	36	0.696	00.274 – 1.766	0.4449

* p. value < 0.05 (significant)
HR: Hazard Ratio
CI: 95% confidence Interval

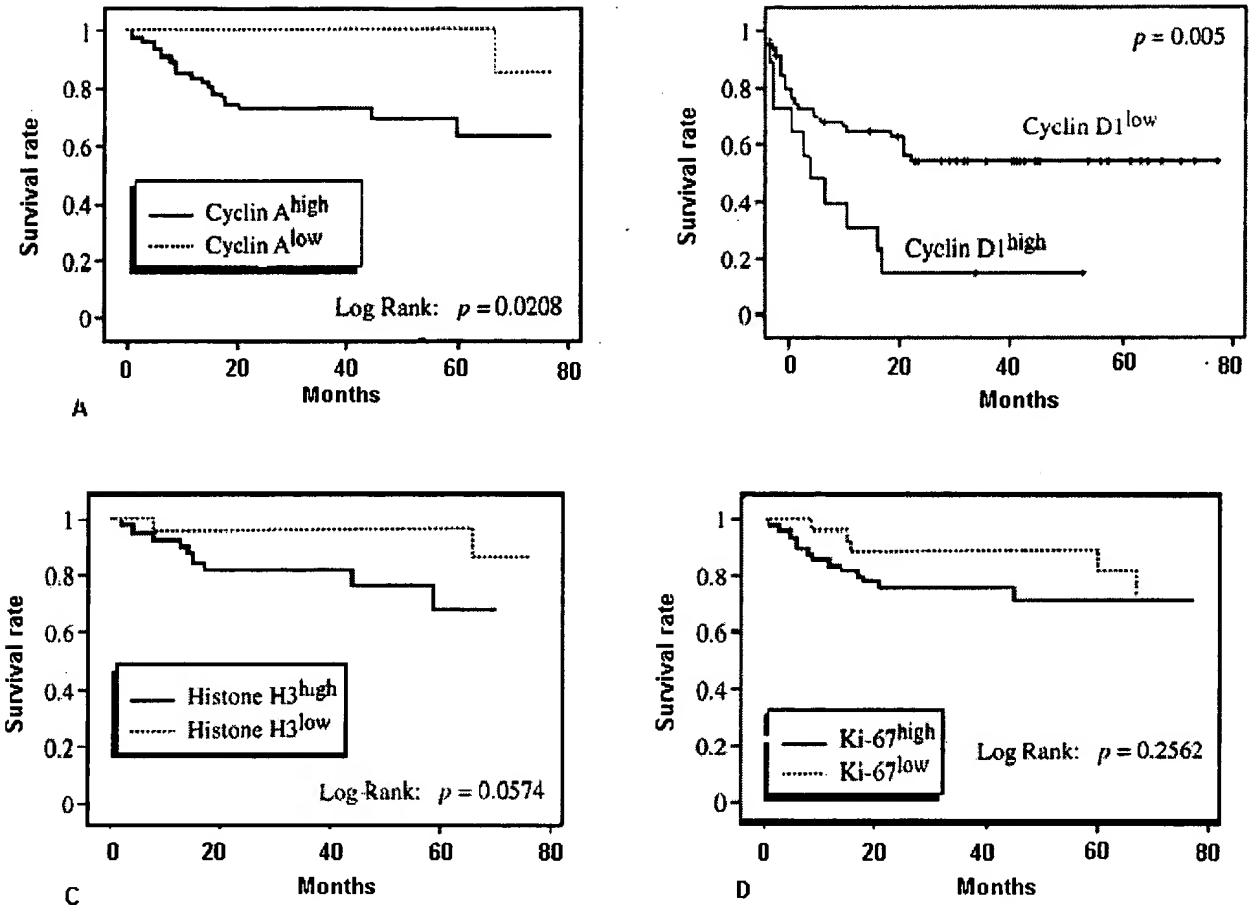

Figure 5
Kaplan-Meier survival curves for colorectal carcinoma. Overall survival is significantly lower in patients with (a) *cyclin A* and (b) *cyclin D1* overexpression. Patients with high SI for *histone H3* mRNA have poorer prognosis but this was not statistically significant (c). No significant difference was present between patients with high *Ki-67* SI and those with low *Ki-67* SI (d).

ate regression analysis revealed that lymph node metastasis, *cyclin A* and *cyclin D1* overexpression were independent negative prognostic factors after adjustment for the depth of tumor invasion, age and sex of the patient (Table 4).

Discussion

The proliferative activity of CRC cells has been investigated in several studies either by immunohistochemical determination of cell proliferation index using antibodies to some types of *cyclins* or by flowcytometric determination of the SPF of the cell cycle [8]. Although Leach et al. [17] did not find *cyclin D1* gene amplification in a panel of 47 CRC cell lines; its protein was overexpressed in about 30% of CRC cases that were included in the studies of Bartakova et al. [6] and Arber et al. [18]. In the former study [6]*cyclin D1* was aberrantly accumulated in a significant subset of human CRC cases and the cell lines derived from these cases were dependent on *cyclin* in their cell cycle progression. In the second study [18], overexpression of *cyclin D1* was detected in 30% of adenomatous polyps indicating that overexpression is a relatively early event in colon carcinogenesis which is possibly responsible for the pathological changes in the mucosa preceding neoplastic transformation. More recently, Holland et al. [19], Pasz-Walczak et al. [20] and Utsunomiya et al. [21] reported up-regulation of *cyclin D1* in 58.7%, 100% and 43% of their studied cases respectively.

Table 4: Multivariate analysis ofthe relationship between survival and thetested markers

PredictiveVariables	HR	CI	P
Cyclin D1	10.864	1.055 – 86.250	0.03*
(baseline < 6.1)	-	-	-
Cyclin A	13.886	1.012 – 190.579	0.0490*
(baseline < 10.5)	-	-	-
Positive Lymph node metastasis	3.921	1.057 – 14.472	0.0410*
Stage IV	3.411	1.048 – 12.083	0.03*
Depth of invasion			
T3, T4	5.408	0.449 – 65.080	0.1836
Age (years)			
≥50	1.996	0.678 – 5.878	0.2310
Sex	0.910	0.315 – 2.358	0.8453

p. value < 0.05 (significant)
HR: Hazard Ratio
CI: 95% confidence Interval

In the present study, up-regulation of *cyclin D1* was detected in 68.3% of the cases. The SI was significantly higher in carcinomas than in normal colorectal mucosa and in poorly-differentiated adenocarcinomas it was approximately twice that of other histological types. Amplification and/or overexpression of *cyclin D1* significantly correlated with deeply invasive tumors and positive lymph node metastasis. Our results in this regards are consistent with previous studies [8,22]. In 2001, Holland et al. [19]. demonstrated that deregulation of *cyclin D1* and $p21^{waf}$ proteins are important in colorectal tumorigenesis and have implications for patient prognosis. Similarly McKay et al. [23] found that *cyclin D1* was the only protein in their panel (*cyclin D1, p53, p16, Rb-1, PCNA and p27*) that correlated with improved outcome in CRC patients. However, few studies failed to detect any correlation between *cyclin D1* overexpression and the clinicopathological factors in CRC [6,18]. This controversy in results could partially be explained by the difference in the sampling of studied cases. The present study included 24 cases of poorly differentiated adenocarcinoma, which is not common in other studies of CRC in western countries. This was possible because the majority of CRC cases diagnosed in Egypt are of high histological grade [3]. The correlation between *cyclin D1* overexpression and the high histological grade was also reported in other tumor types including non-small cell lung carcinomas [24] and squamous cell carcinomas of the larynx [16]. Another possible explanation for the observed controversy in the results of different studies is the detection method used.

In the present work, overexpression of *cyclin D1* was more common than gene amplification of the *PRAD-1/cyclin D1* gene with a 63.6% concordance. This was similarly reported by Bartakova et al. [6] who mentioned that there is a subset of CRC cases in which *cyclin D1* is overexpressed without *PRAD-1/cyclin D1* gene amplification. Consistent with this hypothesis are reports of elevated *cyclin D1* mRNA levels and immunohistochemically detectable accumulation of the protein in over one third of breast cancer cases at a frequency significantly higher than that deduced from DNA amplification studies [9,25]. These data imply that mechanisms other than gene amplification can also lead to deregulation and accumulation of *cyclin D1* in solid tumors.

So far, several studies were done to reveal the prognostic significance of *cyclin D1* overexpression in various carcinomas, including CRC [22]. However, these studies yielded conflicting results which could be attributed to organ heterogeneity. In our study, patients with tumors that exhibited *cyclin D1* overexpression tended to have poor prognosis.

It was reported that, patients with *cyclin A* positive carcinomas had significantly shorter median survival times. Handa et al. [8] were able to detect *cyclin A* overexpression in 77% of their CRC cases. They also demonstrated that, *cylcin A* could be used as a prognostic factor of CRC. More recently, Habermann et al. [26] studied cases of ulcerative colitis with and without an associated adenocarcinoma for the presence of *cyclin A* overexpression. They found that, *cyclin A* overexpression was higher in cases of ulcerative colitis with adenocarcinomas than in those without adenocarcinomas. Consequently, they concluded that, *cyclin A* could be used for monitoring ulcerative colitis patients and for the early detection of an emerging carcinoma in this high risk group of patients.

In our study, *cyclin A* was detected in 80% of the patients and Cox regression analysis showed that it could be used as a prognostic marker in CRC in addition to *cyclin D1*.

It would have been useful if we assessed the expression level of *cyclin A* by another technique (DNA amplification). This would have added more information regarding the gene status on one hand and confirmed the results of IHC on the other hand. Unfortunately, this was not possible because in most of the cases included in the present work, the extracted DNA was not sufficient to study *cyclin amplification* after the assessment of *cyclin D1*.

In 1996, Nagao et al. [11] reported that *histone H3* labeling index significantly correlated with ki-67 immunostaining and was high in poorly differentiated human hepatocellular carcinoma. This was similarly reported in the present work since we found a significant correlation between the SI of *histone H3* and *Ki-67*. However, no

statistically significant correlation was found between *histone H3* SI and any of the studied clinicopathological factors.

Although *Ki-67* immunostaining reflects the proliferative activity of CRC, it has not been recognized as a significant prognostic factor in this type of tumors [27,28]. However, Suzuki at al. [29] found a significant correlation between *Ki-67* labeling index and local invasion of CRC. In the present study there was a significant relationship between the SI of *Ki-67*, tumor size and grade. However, Kaplan-Meier survival curves showed no significant difference in survival rates between patients with- and without overexpression of *Ki-67*.

Conclusions

Our results demonstrate that *cyclin D1, cyclin A, histone H3* and *Ki-67* are overexpressed in a subset of CRC, however only *cyclin D1* and *cyclin A* overexpression correlates with poor differentiation and tumor progression. This indicates the superiority of *cyclin A* and *cyclin D1* as indicators of poor prognosis compared to *Ki-67* and *histone H3* mRNA in CRC. *Cyclin A and D1* could therefore be considered significant, independent prognostic factors in CRC patients. These findings are especially important in stage II patients since 25–30% of those patients have poor prognosis in spite of being node-negative. However, the standard clinicopathologic prognostic factors can not identify this subset accurately and therefore; there is a great demand for more accurate, individually-based, biological prognostic parameters that help in detecting this high risk group of patients who can benefit from an adjuvant therapy. If the findings of the present study are confirmed in a larger study, evaluation of *cyclin A and D1* may be applicable to clinical management of CRC, allowing the identification of patients with poor prognosis.

Competing interests

The author(s) declare that they have no competing interests.

List of abbreviations

CRC – Colorectal cancer

OS – overall survival

SI – staining index

SPF – S phase fraction

ISH – in situ hybridization

m – muscularis mucosa

sm – invasion of the sub mucosa

Authors' contributions

BA and ZA-R carried out the molecular genetic studies, designed, coordinated the study and drafted the manuscript. BA and El-HS carried out all the histopathological and immunohistochemical studies. El-SA participated in molecular genetic studies and drafted the manuscript. MM coordinated the study. El-SM carried out all the patient clinical data. All authors read and approved the final manuscript

References

1. Jiang GL, Huang S: **Adenovirus expressing RIZ1 in tumor suppressor gene therapy of microsatellite unstable colorectal cancers.** *Cancer Res* 2001, **61**:1796-1798.
2. Soliman AS, Bondy ML, Levin B, Hamza MR, Ismail K, Ismail S, Hammam HM, El-Hattab O, Kamal SM, Soliman AG, Dorgham LA, McPherson RS, Beasley RP: **Colorectal cancer in Egyptian patients under 40 years of age.** *Int J Cancer* 1997, **71**:26-30.
3. Soliman AS, Bondy ML, Guan Y, El-Badawy S, Mokhtar N, Bayomi S, Raouf AA, Ismail S, McPherson RS, Abdel-Hakim TF, Beasley PR, Levin B, Wei Q: **Reduced expression of mismatch repair genes in colorectal cancer patients in Egypt.** *Int J Oncol* 1998, **12**:1315-1319.
4. Cordon-Cardo C: **Mutations of cell cycle regulators. Biological and clinical implications for human neoplasia.** *Am J pathol* 1995, **147**:545-560.
5. Hunter T, Pines J: **Cyclins and cancer. II. Cyclin D and CDK inhibitors come of age.** *Cell* 1994, **79**:573-528.
6. Bartkova J, Lukas J, Strauss M, Bartek J: **The *PRAD-1/cyclin D1* oncogene product accumulates aberrantly in a subset of CRCs.** *Int J Cancer* 1994, **58**:568-573.
7. Motokura T, Arnold A: **Cyclins and oncogenesis.** *Biochim Biophys Acta* 1993, **1155**:63-78.
8. Handa K, Yamakawa M, Takeda H, Kimura S, Takahashi T: **Expression of the cell cycle markers in colorectal carcinoma: Superiority of cyclin A as an indicator of poor prognosis.** *Int J cancer* 1999, **84**:225-233.
9. Gillett C, Fantl V, Smith R, Fisher C, Bartek J, Dickson C, Barnes D, Peters G: **Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining.** *Cancer Res* 1994, **54**:1812-1817.
10. Gown AM, Jiang JJ, Matles H, Skelly M, Goodpaster T, Cass L, Reshatof M, Spaulding D, Coltrera DM: **Validation of the S-phase specificity of histone (H3) in situ hybridization in normal and malignant cells.** *J Histochem Cytochem* 1996, **44**:221-226.
11. Nagao T, Ishida Y, Kondo Y: **Determination of S-phase cells by in situ hybridization for histone H3 mRNA in hepatocellular carcinoma: correlation with histological grade and other cell proliferative markers.** *Mod Pathol* 1996, **9**:99-104.
12. Chou MY, Chang AL, McBride J, Donoff B, Gallagher GT, Wong DT: **A rapid method to determine proliferation patterns of normal and malignant tissues by H3 mRNA in situ hybridization.** *Am J Pathol* 1990, **136**:729-733.
13. Sobin LH, Wittekind C: **TNM classification of malignant tumors.** 5th edition. John Wiley, New York; 1997.
14. King RJ, Coffer AI, Gilbert J, Lewis K, Nash R, Millis R, Raju S, Taylor RW: **Histochemical studies with a monoclonal antibody raised against a partially purified soluble estradiol receptor preparation from human myometrium.** *Cancer Res* 1985, **45**:5728-5733.
15. Slebos RJ, Boerrigter L, Evers SG, Wisman P, Mooi WJ, Rodenhuis S: **A rapid and simple procedure for the routine detection of ras point mutations in formalin-fixed, paraffin-embedded tissues.** *Diag Moln Path* 1992, **1**:136-141.
16. Jares P, Fernandez P, Campo E, Nadal A, Bosch F, Aiza G, Nayach I, Traserra J, Cardesa A: ***PRAD-1/cyclin D1* gene amplification correlates with messenger RNA overexpression and tumor progression in human laryngeal carcinomas.** *Cancer Res* 1994, **54**:4813-4817.
17. Leach FS, Elledge SJ, Sherr CJ, Willson JK, Markowitz S, Kinzler KW, Vogelstein B: **Amplification of cyclin genes in colorectal carcinomas.** *Cancer Res* 1993, **53**:1986-1989.

18. Arber N, Hibshoosh H, Moss SF, Sutter T, Zhang Y, Begg M, Wang S, Weinstein IB, Holt PR: **Increased expression of cyclin D1 is an early event in multistage colorectal carcinogenesis.** *Gastroenterology* 1996, **110**:669-674.
19. Holland TA, Elder J, McCloud JM, Hall C, Deakin M, Fryer AA, Elder JB, Hoban PR: **Subcellular localization of cyclin D1 protein in colorectal tumors is associated with p21 (WAF1/CIP1) expression and correlates with patient survival.** *Int J Cancer* 2001, **95(5)**:302-306.
20. Pasz-Walczak G, Kordek R, faflik M: **P21(WAF1) expression in colorectal cancer: correlation with p53 and cyclin D1 expression, clinicopathological parameters and prognosis.** *Pathol Res Pract* 2001, **197(10)**:683-689.
21. Utsunomiya T, Doki Y, Takemoto H, Shiozaki H, Yano M, Sekimoto M, Tamura S, Yasuda T, Fujiwara Y, Monden M: **Correlation of beta-catenin and cyclin D1 expression in colon cancers.** *Oncology* 2001, **61(3)**:226-233.
22. Maeda K, Chung YS, Kang SM, Ogawa M, Onoda N, Nakata B, Nishiguchi Y, Ikehara T, Okuno M, Sowa M: **Overexpression of cyclin D1 and p53 is associated with disease recurrence in colorectal adenocarcinoma.** *Int JCancer* 1997, **74**:310-315.
23. McKay JA, Douglas JJ, Ross VG, Curran S, Loane JF, Ahmed FY, Cassidy J, McLeod HL, Murray GI: **Analysis of key cell cycle checkpoint proteins in colorectal tumors.** *J Pathol* 2002, **196**:386-393.
24. Mate JL, Ariza A, Aracil C, Lopez D, Isamat M, Perez-Piteira J, Navas-Palacios JJ: **Cyclin D1 overexpression in non-small cell lung carcinoma: correlation with Ki-67 labeling index and poor cytoplasmic differentiation.** *J Pathol* 1996, **180**:395-399.
25. Buckley MF, Sweeney KJ, Hamilton JA, Sini RL, Manning DL, Nicholson RI, DeFazio A, Watts CK, Musgrove EA, Sutherland RL: **Expression and amplification of cyclin genes in human breast cancer.** *Oncogene* 1993, **8**:2127-2133.
26. Habermann J, Lenander C, Roblick UJ, Kruger S, Ludwig D, Alaiya A, Freitag S, Dumbgen L, Bruch HP, Stange E, Salo S, Tryggvason K, Auer G, Schimmelpenning H: **Ulcerative colitis and colorectal carcinoma: DNA profile, laminin-5 gamma 2 chain and cyclin A expression as early markers for risk assessment.** *Scand J Gastroenterol* 2001, **36**:751-758.
27. Kubota Y, Petras RE, Easley KA, Bauer TW, Tubbe RR, Fazio VW: **Ki-67-determined growth fraction versus standard staging and grading parameters in colorectal carcinoma. A multivariate analysis.** *Cancer* 1992, **70**:2602-2609.
28. Shain AA, Ro JY, Brown RW, Ordonez NG, Cleary KR, El-Naggar AK, Wilson P, Ayala AG: **Assessment of Ki-67-derived tumor proliferative activity in colorectal adenocarcinomas.** *Mod Pathol* 1994, **7**:17-22.
29. Suzuki H, Matsumoto K, Terabe M: **Ki-67 antibody labeling index in colorectal carcinoma.** *J clin Gastroenterol* 1992, **15**:317-320.

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Correlation of amplification and overexpression of the c-*myc* oncogene in high-grade breast cancer: FISH, *in situ* hybridisation and immunohistochemical analyses

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In this study, we analysed gene amplification, RNA expression and protein expression of the c-*myc* gene on archival tissue specimens of high-grade human breast cancer, using fluorescent *in situ* hybridisation (FISH), nonradioactive *in situ* hybridisation and immunohistochemistry. The specific question that we addressed was whether expression of c-*Myc* mRNA and protein were correlated with its gene copy amplification, as determined by FISH. Although c-*Myc* is one of the most commonly amplified oncogenes in human breast cancer, few studies have utilised *in situ* approaches to directly analyse the gene copy amplification, RNA transcription and protein expression on human breast tumour tissue sections. We now report that by using the sensitive FISH technique, a high proportion (70%) of high-grade breast carcinoma were amplified for the c-*myc* gene, irrespective of status of the oestrogen receptor. However, the level of amplification was low, ranging between one and four copies of gene gains, and the majority (84%) of the cases with this gene amplification gained only one to two copies. Approximately 92% of the cases were positive for c-*myc* RNA transcription, and essentially all demonstrated c-myc protein expression. In fact, a wide range of expression levels were detected. Statistically significant correlations were identified among the gene amplification indices, the RNA expression scores and protein expression scores. c-*myc* gene amplification, as detected by FISH, was significantly associated with expression of its mRNA, as measured by the intensity of *in situ* hybridisation in invasive cells ($P = 0.0067$), and by the percentage of invasive cells positive for mRNA expression ($P = 0.0006$). c-*myc* gene amplification was also correlated with the percentage of tumour cells which expressed high levels of its protein, as detected by immunohistochemistry in invasive cells ($P = 0.0016$). Thus, although multiple mechanisms are known to regulate normal and aberrent expression of c-*myc*, in this study, where *in situ* methodologies were used to evaluate high-grade human breast cancers, gene amplification of c-*myc* appears to play a key role in regulating expression of its mRNA and protein.
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Genetics and Genomics

The c-*myc* oncogene has been shown to be amplified and/or overexpressed in many types of human cancer (Marcu *et al*, 1992; Nass and Dickson, 1997; Nesbit *et al*, 1999; Liao and Dickson, 2000). Numerous experiments *in vivo* have also causally linked aberrant expression of this gene to the development and progression of cancer in different body sites (Marcu *et al*, 1992; Nass and Dickson, 1997; Nesbit *et al*, 1999; Liao and Dickson, 2000). However, several critical issues regarding the significance of c-*myc* in human cancer still remain obscure. First, even for a given type of malignancy, the frequencies of the alterations of c-*myc* at the cytogenetic and expression levels vary greatly from one report to another (Liao and Dickson, 2000). For instance, the frequencies of its amplification, mRNA and protein overexpression in breast cancer vary between 1–94, 22–95 and roughly 50–100%, respectively, among different reports (Liao and Dickson, 2000). Thus, it is still unclear to what extent this gene is altered at the cytogenetic level and at different expression levels in breast carcinoma.

One controversial issue pertains to the prognostic value of c-*myc* gene alterations in cancer. The central role of c-Myc protein in accelerating cell proliferation, documented by many early studies, has led to a general concept for many types of cancer that amplification or overexpression of this gene may be associated with a more aggressive tumour and a poorer patient survival (Berns *et al*, 1992; Marcu *et al*, 1992; Sato *et al*, 1995; Nass and Dickson, 1997; Nesbit *et al*, 1999; Visca *et al*, 1999; Liao and Dickson, 2000). However, many reports have shown an opposite correlation (Sikora *et al*, 1985, 1987; Watson *et al*, 1986; Polacarz *et al*, 1989; Voravud *et al*, 1989; Williams *et al*, 1990; Melhem *et al*, 1992; Pietilainen *et al*, 1995; Diebold *et al*, 1996; Smith and Goh, 1996; Augenlich *et al*, 1997; Bieche *et al*, 1999), while other studies do not support either of these conclusions. For instance, gene amplification or overexpression of c-Myc protein has also been shown to associate with a better tumour differentiation or a better

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patient survival for cancer of the testis, ovary, bile ducts, colon and breast (Sikora *et al*, 1985, 1987; Watson *et al*, 1986; Polacarz *et al*, 1989; Voravud *et al*, 1989; Williams *et al*, 1990; Melhem *et al*, 1992; Pietilainen *et al*, 1995; Diebold *et al*, 1996; Smith and Goh, 1996; Augenlich *et al*, 1997; Bieche *et al*, 1999). This controversy does not appear to be related completely to the cancer type, since both positive (Berns *et al*, 1992; Visca *et al*, 1999) and negative (Williams *et al*, 1990; Melhem *et al*, 1992; Pietilainen *et al*, 1995; Smith and Goh, 1996; Augenlich *et al*, 1997; Bieche *et al*, 1999) correlations have been reported for colon cancer and breast cancer. More interestingly, c-Myc overexpression has been shown to predict a poorer prognosis for cutaneous melanoma, but a favourable outcome for uveal melanoma (Grover *et al*, 1997; Chana *et al*, 1998a, b, 1999; Grover *et al*, 1999). These data indicate different roles of c-Myc, even in the same type of tumour, perhaps depending upon different tissue microenvironments.

Another controversial issue concerns the nuclear–cytoplasmic localisation of c-Myc. Studies of neoplasms of the colon, testis, ovary and liver have shown that predominantly nuclear localisation of c-Myc tends to occur in benign lesions, while cytoplasmic localisation tends to occur in more malignant tumours (Sikora *et al*, 1985; Sundaresan *et al*, 1987; Melhem *et al*, 1992; Sasano *et al*, 1992; Yuen *et al*, 2001). Whether these patterns of subcellular localisation of c-Myc tend to reflect the malignant status of breast cancer remains an enigma.

A recent study of the impact of DNA amplification on gene expression patterns in breast cancer used mRNA and DNA from 14 breast cancer cell lines. Analysis was conducted with a 13 000 cDNA clone array for gene expression measurement and a Comparative Genomic Hybridisation (CGH) microarray for gene copy number measurements. This study also included known breast cancer genes, such as c-*myc*, *HER/2-neu* and *aib1* (Hyman *et al*, 2002). Interestingly, 44% of the most highly amplified genes were also overexpressed at the mRNA level. Consistent with this pattern, c-*Myc* gene copy number and its expression levels showed a statistically significant ($\alpha = 0.020$) correlation in this microarray study of breast cancer cell lines. Another study, by Pollack and colleagues, used microarray analysis and BAC array CGH of RNA and DNA (respectively) extracted from intermediate grade human breast tissues, and tested for amplification and expression of c-Myc (among other genes). This study demonstrated that two out of 37 specimens were both amplified and overexpressed, while others were either amplified or overexpressed, but not both. The authors of this study suggested that contaminating stromal tissue may compress the fluorescence ratios leading to underestimates of gene amplification and overexpression (Pollack *et al*, 2002).

To more clearly address the importance of gene amplification and expression of c-Myc in human breast cancer, we used *in situ* methodologies, which can clearly distinguish stromal and carcinoma components. We studied the amplification and over expression of the c-*myc* gene with fluorescent *in situ* hybridisation (FISH), non-radioactive *in situ* hybridisation (ISH) and immunohistochemical (IHC) approaches on paraffin-embedded biopsy sections of untreated, high-grade breast cancer. It was observed that 70, 92 and 70% of the cancer cases exhibited c-*myc* gene amplification, its mRNA overexpression and its protein over expression, respectively. In most of the cases (84%) that showed gene amplification, the c-*myc* gene gained only one to two copies, which is consistent with c-*myc* FISH data from other studies. Unlike some oncogenes, such as N-*myc*, which typically demonstrates gene amplification copy numbers of greater then 10 in neuroblastoma, and *HER-2/neu* (Sartelet *et al*, 2002), whose copy numbers range up to 14–40 in breast carcinomas (Isola *et al*, 1999), gene copy numbers of c-*myc* are not as greatly increased. In the study noted earlier, using breast cancer cell line CGH array and cDNA microarray expression analysis, it was demonstrated that the most dramatically increased expression levels were associated with large gene copy number increases, although low-level gains and losses had a significant influence on gene expression dysregulation (Hyman *et al*, 2002). Only one study has been published (Pollack *et al*, 2002) that has begun to determine if these findings are directly relevant to actual human breast tumour tissues, since many of the genetic changes in tissue culture cell lines are more extreme than those displayed in primary tumour material. Furthermore, the relationships among gene amplification, mRNA expression and c-Myc protein expression were not explored in prior human breast cancer cell line and tumour tissue studies (Hyman *et al*, 2002; Pollack *et al*, 2002).

In our human breast tumour tissue study, a high correlation was found between c-*myc* FISH and ISH, for both percentage of staining ($P<0.0067$) and intensity positive cells ($P<0.0006$). In addition, c-*myc* gene copy amplification by FISH was correlated with c-Myc protein expression positive cells by IHC ($P<0.0016$). These results support the idea that c-Myc overexpression of both mRNA and protein is related to the copy number of the *c-myc* DNA amplification. We show in this study that amplification and overexpression of c-Myc occur with high frequency in high-grade human breast cancer tissues.

MATERIALS AND METHODS

Materials

Formalin-fixed, paraffin-embedded tissue blocks of breast carcinoma and normal breast tissue were obtained from the Histopathology and Tissue Shared Resource at the Lombardi Comprehensive Cancer Center (LCCC), at Georgetown University Medical Center. The criteria for tumour selection were the following: negative progesterone receptor status, metastases to auxiliary lymph nodes and high grade (Elston Score >7). The oestrogen receptor status of the tumours was known from archived pathology reports. The parameters were chosen from our prior meta-analysis (Deming *et al*, 2000), as indications of a high likelihood of c-*myc* gene amplification. Normal breast tissue specimens were from reduction mammoplasty. Serial sections (5 μm) for FISH, ISH and IHC were prepared by the LCCC Histopathology and Tissue Shared Resource.

FISH

A dual-label FISH technique was used (Jenkins *et al*, 1997). Slides were baked overnight at 60°C to assure adherence of the sample. Tissue sections were deparaffinised with two successive, 10 min xylene washes, and then dehydrated in a graded ethanol series of 70, 80 and 95% at room temperature. Samples were then digested with 4% pepsin (Sigma, St Louis, MO, USA) at 45°C for 10 min. DNA probes used were an alpha satellite probe to chromosome 8, labelled with biotin, and a *c-myc* probe, labelled with digoxigenin (Ventana, Tucson, AZ, USA). Codenaturation was performed at 90° for 10 min on a hot plate. Hybridisation was at 37°C for 12–16 h. Detection of signals was accomplished with an antiavidin antibody labelled with Texas Red, and an antidigoxigenin antibody conjugated to fluorescein (Ventana, Tucson, AZ, USA). Slides were postwashed in 2 × SSC at 72°C for 5 min and counterstained with DAPI to visualise cell nuclei. Results were viewed and quantified with a Zeiss Axiophot fluorescence microscope, equipped with appropriate filters and an Applied Imaging Cytovision system (Pittsburgh, PA, USA). In this approach, the c-*myc* unique sequence probe was visualised as a green signal and the control probe for the chromosome 8 centromere was red, thus easily being distinguished when scored.

One serial section from each tumour sample was stained with haematoxylin and eosin and first reviewed by a pathologist (BS), to help identify the tumour area of the section. This procedure ensured that the tumour cells, but not the normal cells, were

counted. Nuclei of up to 50 tumour cells were scored from each FISH-stained section, independently by two investigators. Hybridisation signals were averaged, and the amplification index was presented as the number of c-*myc* signals divided by the number of chromosome 8 centromere signals. A 1.8-fold increase was used as the criterion to judge the presence of c-*myc* gene amplification.

In situ hybridisation

In situ hybridisation (ISH) was carried out with a nonradioactive method, described previously (Liao *et al*, 2000a, b). One serial section from each specimen was hybridised overnight at 60°C with riboprobes, that were *in vitro* transcribed from the antisense or sense strand of an approximately 300 bp cDNA of human c-*myc* (ATCC, Manassas, VA, USA), labelled with digoxigenin-conjugated UTP. The sections were then incubated with an antibody against digoxigenin, followed by incubation with a second antibody conjugated to alkaline phosphatase. The signal was visualised by colour development with 5 bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. All reagents were purchased from Boehringer Mannheim, Indianapolis, IA. To control the signal specificity, two serial sections were mounted on the same slide for hybridisation with the antisense and sense probes, respectively. ISH was given an intensity and percentage scores, based on intensity of positive staining and number of cells staining, respectively. Intensity scores were assigned 0, 1, 2 and 3, and percentage scores were assigned as 1- 1–25, 2- 26–50, 3- 51–75 and 4- 76–100%.

Immunohistochemistry

Immunohistochemical staining (IHC) was performed using an avidin–biotin complex (ABC) method described previously (Liao *et al*, 1998). One serial section of each specimen was deparaffinised and blocked with 3% peroxide. Antigens were retrieved by heating slides in a microwave oven in 50 mM citrate buffer, pH 6.4, at boiling temperature, for 12 min. After blocking with 6% normal goat serum, the section was incubated with a mouse monoclonal antibody to human c-Myc (9E10, Sigma Chemical Company, St Louis, MO, USA) at 1:100 dilution for 2 h, followed by 1 h incubation with a second antibody conjugated with biotin (Vector Laboratories Inc., Burlingame, CA, USA). The section was then incubated with peroxidase-conjugated avidin (Dako, Corporation, Carpinteria, CA, USA) for 30 min, followed by colour development with diaminobenzidine and peroxide. All procedures were carried out at room temperature. To control the signal specificity, serial sections from 10 tumour samples were also stained using an alternate c-Myc antibody (C19 from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1:60 dilution. This antibody resulted in focally positive staining in the tumour, but the staining intensity was weaker. To control the signal specificity, serial sections were made from five selected positive cases which were subjected to the same staining procedure, with a normal mouse IgG to replace the c-Myc antibody. This control staining did not give rise to a signal, demonstrating the specificity of the c-Myc antibody signal. IHC staining was given an intensity and percentage score based upon the intensity of positive staining and number of cells staining. Intensity scores were assigned 0, 1, 2 and 3 and percentage scores were assigned as 1- 1–25, 2- 26–50, 3- 51–75 and 4- 76–100%. Determinations were made of cellular localisation of c-Myc antibody staining to cytoplasm and/or nucleus in normal and invasive cells within each breast tumour specimen.

Statistical analyses

For each analysis of gene copy amplification (FISH), mRNA expression (ISH) and protein expression (IHC), all cases were first grouped as positive or negative to calculate the percentages of positive cases and negative cases, as described (Zar, 1974). Fisher's exact test was used to compare percentages, and two-sample *t*-test or Wilcoxon rank test was used to compare average scores. Both ISH and IHC were given intensity and percentage scores, based on intensity of positive staining and number of cells staining, respectively. As noted earlier, intensity scores were assigned 0, 1, 2 and 3 and percentage scores were assigned as 1- 1–25, 2- 26–50, 3- 51–75 and 4- 76–100%. A score of >2 for either intensity of staining or percentage of cells positive by ISH was assigned as high. For IHC, an intensity score of >1 was assigned as high and a percentage score of >3 was categorised as high. Each amplification index was paired with its corresponding mRNA expression score to calculate the coefficient *r*. The same method was used to estimate the association of the amplification indices with the c-Myc protein expression levels, and the association of the mRNA expression levels with the protein expression levels. A *P*-value of 0.05 or less was used to determine the statistical significance in all analyses. In all, 54 pairs of normal *vs* invasive tissues were analysed using McNemars χ^2 test to determine if there was a difference in cellular localisation of c-Myc antibody signal to nuclear or cytoplasmic compartments.

RESULTS

FISH analysis of gene amplification

Amplification of the c-*myc* gene was measured by a FISH test in 46 cases of breast cancer; Figure 1 demonstrates cells with no amplification (one copy of c-*myc* /one copy of chromosome 8 centromere, and a moderate amplification a 3/1 ratio). Amplification was calculated by the number of c-*myc* signals divided by the number of chromosome 8 alpha satellite signals. A 1.8-fold increase cut-off was used to judge gene amplification. As shown in Table 1, 32 out of 46 (70%) cases were gene amplified for c-*myc*, whereas only 30% (14/46) of the cases showed amplification indices lower than the cut-off value. The amplification indices for most (84%, or 27/32) cases with gene amplification, ranged between 1.8- and three-fold, indicating that the locus gained up to two copies of c-*myc* in the majority of the cases. The percentage of cases with gene gains of three copies or higher was 11% (five out of 46) of total cases analysed, or near 16% (five out of 32) of the cases with gene amplification, including one case (2% of total cases or 3% of the cases with gene amplification) with the highest index of 5 (a gain of four copies).

In all, 28 of the breast carcinomas in this study were ER negative, and 14 were ER positive. The average c-*myc* gene amplification score was 1.896 (s.e. = 0.196) for ER positive and 2.201 (s.e. = 0.157) for ER negative. Although ER-negative tumours had a slightly higher average c-*myc* score, the difference was not statistically significant (two-sided $P = 0.252$ from two-sample *t*-test and 0.251 from Wilcoxon rank test), consistent with the results of our prior meta-analysis of the literature (Deming *et al*, 2000).

In situ hybridisation analysis of c-*myc* mRNA expression

A total of 51 breast cancer samples were studied for c-Myc mRNA expression, with non radioactive *in situ* hybridisation (ISH). ISH results were assigned intensity and percentage scores based upon signal intensity of positive staining and number of cells staining within the sample, respectively. As shown in Table 2, 86% (44 out of 51) tumours were scored as high in intensity, and 92% (47out of 51) had more than 51% positive cells, also considered as highly increased c-Myc expression. mRNA expression was heterogeneous in the breast tumour tissue, and no morphologic subtype was predominant in the high or low categories. One case showed no c-Myc ISH staining. In 79% (38/48) of cases, epithelia in normal mammary glands adjacent to the tumour also showed a high

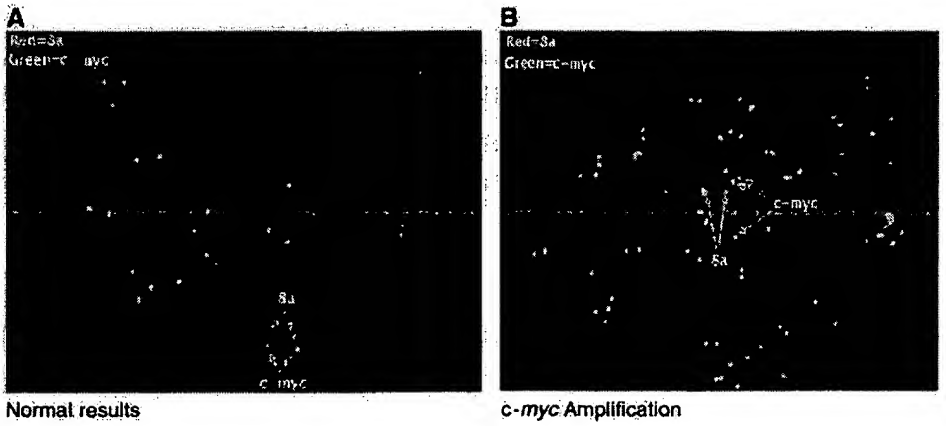

Figure 1 FISH analysis of c-*myc* amplification in tumour cells from breast tumour tissue sections. FISH probe for human c-*myc* unique-sequence is seen as green, while the normal control signal, a centromeric probe signal for chromosome 8 is shown in red. The nuclei of tumour cells were visualised by DAPI counter-staining. (**A**) 1 : 1 copy ratio of c-*myc* to chromosome 8 (c-*myc*/8 centromere), indicating no amplification of c-*myc* in tumour cells. (**B**) 1 : 3 copy ratio of c-*myc* to chromosome 8 (c-*myc*/8 centromere), a moderate amplification of the c-*myc* gene.

Table 1 c-*myc* gene copy amplification analysis by FISH in poor prognosis human breast tumour samples

Amplification index (#c-myc signals/# control signals)+	Percentage of samples with FISH ratios in each category	
1.0–1.7	30%	14 out of 46
1.8–1.99	20%	Nine out of 46
2.0–2.9	39%	18 out of 46
>3.0	11%	Five out of 46

Analysis was conducted on 46 individual paraffin-embedded tissue samples with negative progesterone receptor status, positive lymph node involvement and high tumour grade. +Normal control ratio is 1.

intensity of staining. In three cases, no staining was seen in the normal terminal duct lobular units. Figure 2 shows representative fields of high, medium and low *c-myc* mRNA expression levels in invasive ductal carcinoma samples.

Association of FISH and ISH

c-Myc scores were dichotomised as binary variables (high or low), and a score of 2 or higher was categorised as high on ISH. A score higher than median was categorised as high from FISH studies. These dichotomised scores are depicted in Table 3. A Fisher's exact test was performed for comparing binary responses to see if there was any association between FISH and ISH. It was found that the FISH score was significantly associated with percentage of staining in the invasive cells ($P=0.0067$, two-sided McNemar's test) and also with the intensity score on ISH ($P=0.0006$, two-sided).

Immunohistochemical staining of c-Myc proteins

In total, 51 breast carcinomas, which were subjected to FISH analysis, and all of which also had been analysed for c-*myc* mRNA by *in situ* hybridisation, were also analysed for the expression of c-Myc protein, using immunohistochemical staining with the 9E10 antibody. IHC results were assigned an intensity and percentage score based on intensity of positive staining and number of cells staining, respectively. Intensity scores were assigned 0, 1, 2 and 3 and percentage scores were assigned as 0, 1- 0–25, 2- 26–50, 3- 51–75 and 4- 76–100. For IHC, an intensity score of >1 was assigned as high and a percentage score of >3 was categorised as high. Figure 2 shows examples of high, medium and low levels of c-myc antibody staining in invasive ductal carcinoma samples. In 34 cases, normal tissue was seen; 30 of these showed cytoplasmic staining and 22 had nuclear staining in terminal ductal lobular units. In all, 12 cases showed 1+, 14 cases 2+ and four cases 3+ cytoplasmic staining. *In situ* hybridisation revealed positive staining in 46 out of 49 cases with normal tissue. Seven cases showed 1+, 13 cases showed 2+ and 26 cases showed 3+ staining by ISH. Both immunohistochemistry and *in situ* hybridisation showed diffuse positivity in adipocytes.

Table 4 shows the staining pattern for the cohort. In all, 70% (36 out of 51) of cases showed high intensity of staining for c-Myc protein, while 85% (29 out of 34) of cases with detectable staining had more than 76% positive cells, also considered as high expression. To verify the staining specificity, serial sections from 10 tumour specimens that were positive for 9E10 antibody were also stained using the C19 rabbit polyclonal anti-c-Myc antibody. Results revealed a staining pattern similar to 9E10. However, the staining intensity with C19 was weaker than 9E10. The specificity of these two antibodies was verified by Western blots in previous studies (Persons *et al*, 1997; Liao *et al*, 2000b). Figure 2 shows results of c-Myc *in situ* hybridisation and immunohistochemistry studies on samples considered to demonstrate low, moderate and high levels of c-Myc expression. Analysis of c-Myc protein localisation results in the nucleus or cytoplasmic compartments of normal and invasive cells within the tumours revealed that nuclear staining was positive in 41% of normal cells, compared to 22% of invasive cells (statistical significance at $P=0.01$ by McNemar's two-sided χ^2 test). The increase in relative cytoplasmic localisation of c-Myc protein, comparing normal (53.7%), to invasive cells (61.1%) was not significantly different. Thus, the data are consistent with partial exclusion of c-Myc from the nuclei of invasive breast cancer cells.

The FISH score was significantly associated with the percentage positivity of invasive cells, as seen on IHC studies of c-Myc. However, 40% of tumours displayed a low index of c-*myc* gene amplification, but still expressed high levels of c-Myc protein (Table 6), indicating the possibility of other mechanisms of over expression unrelated to gene amplification in at least some tumours. The FISH score was not significantly associated with the intensity of IHC staining in the invasive cells (not shown), in contrast to the IHC percentage positivity score.

Table 2 c-myc mRNA *in situ* hybridisation (ISH) results

Staining intensity	0	1	2	3	Percent positivity	1	2	3	4
Number of tumour samples in each category $N=51$	1	6	25	19	Number of tumour samples in each level category $N=51$	1	3	5	42

In all, 51 human high-grade breast carcinomas were analysed to determine the relationships between c-Myc mRNA expression and *c-myc* gene *in situ* hybridisation results. Data are shown in two ways in the above table. First, overall staining intensity of c-Myc-positive cells was scored as 0, 1, 2, 3 (low to high), and the number of tumour samples at each level of staining indicated on the line below. Next, the percentage of tumour cells staining was scored as 0, 1, 2, 3, 4 (low to high %, as discussed in Materials and Methods). The number of tumours at each level of percent cell positivity for c-Myc is then indicated on the line below.

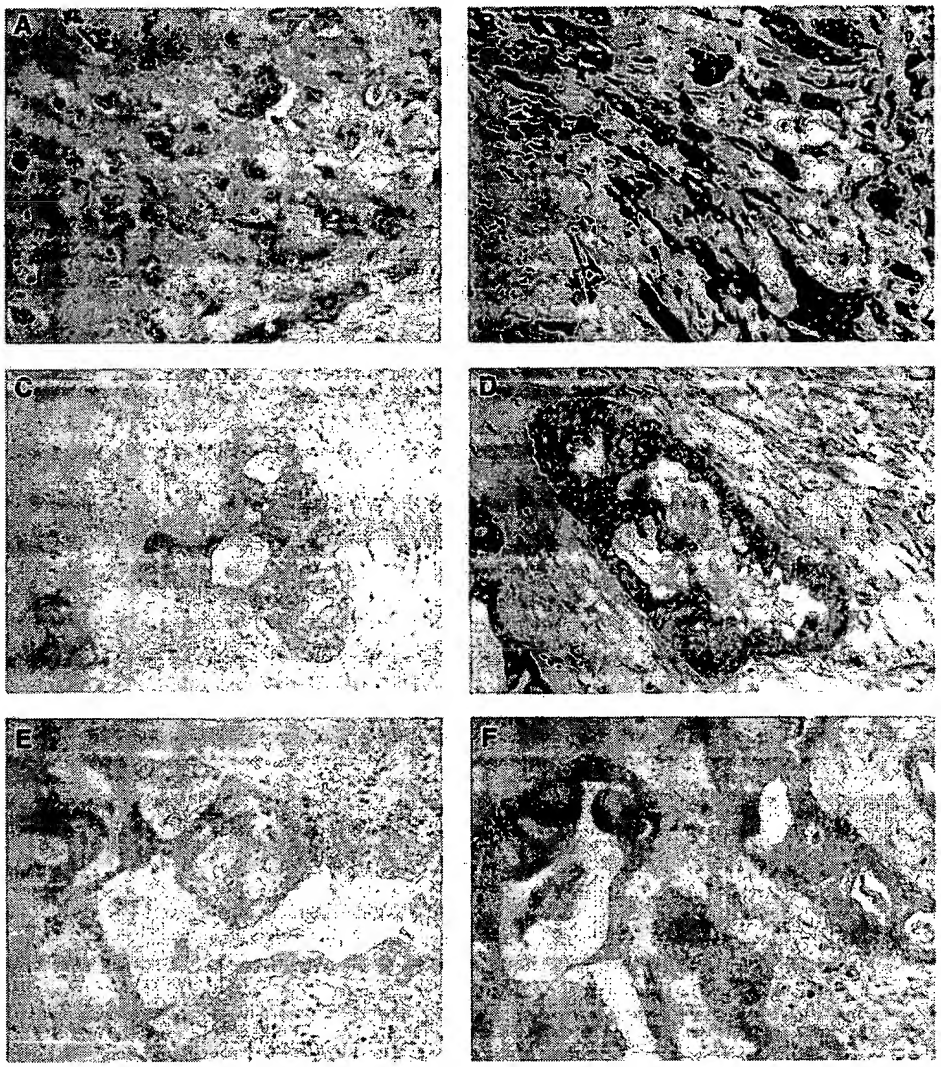

Figure 2 Immunohistochemical staining and *in situ* hybridisation for c-Myc of three sets of invasive ductal carcinoma. (**A**, **C** and **E**) High (3+), intermediate (2+) and low (1+) level of staining by immunohistochemistry for c-Myc. (**B**, **D** and **F**) High (3+), intermediate (2+) and low (1+) level of staining by *in situ* hybridisation.

DISCUSSION

Although there have been many reports on *c-myc* amplification in human breast cancer (Liao and Dickson, 2000), there are only two published studies involving application of the FISH technique to unfixed, frozen sections (Persons *et al*, 1997; Visscher *et al*, 1997), and one prior study using FISH on an archival human tissue microarray (Schraml *et al*, 1999). Another recent study applied FISH to evaluate *c-myc* amplification in ductal carcinoma *in situ* (DCIS) (Aulmann *et al*, 2002). Using the FISH technique on formalin-fixed, paraffin-embedded sections, we now show that 70% of high-grade breast cancer samples bear *c-myc* gene copy amplifications. Interestingly, the above-mentioned study, using FISH and focusing on DCIS, detected amplification of *c-myc* in only 20% of cases, but found a correlation of *c-myc* with increased tumour size and proliferation (Aulmann *et al*, 2002).

The level of amplification of *c-myc* in our study ranged between one and four additional copies of the gene; the majority (84%) of

the cases with the gene amplification gained only one to two copies, also consistent with FISH data reported for c-*myc* copy amplification in human metastatic prostate carcinoma tissues (Jenkins *et al*, 1997). The relationship between the level of c-*myc* gene copy amplification and the level its increased mRNA expression has been examined previously in breast cancer cell lines (Hyman *et al*, 2002). In general, it has been concluded that the two scores coordinate for c-*myc*, as is the case for many breast cancer genes. However, only 44% of the highly amplified genes, in general, showed increased RNA expression, and only 10.5% of the highly overexpressed genes were gene copy-amplified in the cell line study (Hyman *et al*, 2002). Another analysis was conducted to study of relationships between gene amplification and expression of 6095 genes in 37 intermediate grade human breast tumours. This study demonstrated that 62% of the highly amplified genes also showed elevated expression; overall, a two-fold change in DNA copy number was associated with a 1.5-fold change in mRNA levels. Overall, 12% of the variation in gene expression in the breast tumours studied was associated with gene copy number variation (Pollack *et al*, 2002). Further study of additional human breast tumours, at precisely defined grades and stages, will be necessary in order to more fully define the relationships between DNA copy numbers and expression of genes. The studies we report here indicate higher levels of c-Myc gene amplification and expression, than other previous reports in breast cancer. We believe that this is probably the result of our analysis of individual tumour cells in a well-defined set of high-grade breast tumours. Prior c-Myc expression and amplification microarray studies used tumour specimens which contain normal stromal components, potentially underestimating amplification and expression levels of the invasive tumour components (Pollack *et al*, 2002).

Our study reports a percentage of tumours gene amplified for c-*myc* (using FISH in high-grade tumours) that is much higher than the average figure (15.5%) reported in the literature (Isola *et al*, 2002). Most of the prior studies have employed the relatively insensitive Southern blot technique, and were reviewed in a recent meta-analysis (Deming *et al*, 2000). Consistent with this prior literature background, a recent study of 94 lobular and ductal breast cancers assessed amplification of c-*myc* by using a semiquantitative PCR assay and protein expression, with

Table 3 Correlations between c-*myc* gene copy number (FISH) mRNA expression (ISH)

	FISH	
	Low	**High**
(A) ISH (% cells)		
Low	*1*	3
High	*19*	*18*
		$P = 0.0067$
(B) ISH (intensity)		
Low	*2*	*5*
High	*18*	*16*
		$P = 0.0006$

Serial sections of high-grade human breast carcinomas were scored for c-*myc* gene copy number (FISH, Table 1) and mRNA expression (ISH, Table 2). In (A), a positive correlation ($P = 0.0067$) was observed between tumour samples with a high percentage of cells demonstrating mRNA expression and a high c-*myc* gene copy number. A score of 2 or higher was classified as high on ISH, and a score of median or greater was categorised as high on FISH. In (B), a positive correlation ($P = 0.0006$) was shown between a high level of intensity for c-Myc RNA expression a high and c-*myc* gene copy number. Note that a pairwise comparison of FISH and ISH was not possible for all cases, due to incomplete overlap of cases analysed with each assay.

Table 5 Nuclear/cytoplasmic localisation of c-Myc comparing normal and invasive cells

Normal cells (frequency percent)	**Invasive cells (frequency percent)**		**Total**
(A) Nuclear localisation			
	–	+	
–	28	4	32
+	14	8	22
Total	42	12	54
(B) Cytoplasmic localisation			
–	12	13	25
+	9	20	21
Total	21	33	54

In all, 54 pairs (normal *vs* invasive) of tissues were analysed to answer the questions of (1) whether positivity of nuclear cells in normal tissues is different from that in invasive cells, and similarly (2) whether positivity of cytoplasmic cells in normal tissues is different from that in invasive cells. The data are summarised in the above contingency tables. In all, 22 normal cell specimens were positive for c-Myc staining (40.71%), compared to 12 specimens (22.2%) in invasive cells. The difference is statistically significant ($P = 0.01$) by McNemar's χ^2 test (two-sided).

Table 6 Correlation between c-Myc protein expression (IHC) and c-*myc* gene copy number (FISH)

	FISH	
IHC (% cells)	**Low**	**High**
Low	3	0
High	10	15
		$P = 0.0016$

Consecutive serial sections of high-grade human breast tumours were scored for c-*myc* gene copy number or protein expression, by immunohistochemistry (IHC). IHC scores were defined in the Materials and methods section. Data were analysed for correlations between the results. A highly significant correlation was observed between high c-Myc protein expression (IHC) between percent cells positive and high c-*myc* gene amplification (FISH). $P = 0.0016$ from two-sided McNemar's test. Note that for 15 cases, no staining for c-Myc could be detected; these negative cases were not included in the correlation presented, above.

Table 4 c-Myc immunohistochemistry (IHC) results

Staining intensity	**0**	**1**	**2**	**3**	**Percent positivity**	**1**	**2**	**3**	**4**
Number of tumour samples in each category	15	13	20	3	Number of tumour samples in each category	2	2	1	29

In all, 51 high-grade human breast carcinomas were analysed to determine the relationships between c-Myc protein expression and c-*myc* gene *in situ* hybridisation results. Data are shown in two ways in the above table. First, overall staining intensity of c-Myc-positive cells was scored as 0, 1, 2, 3 (low to high), and the number of tumour samples at each level of staining is indicated on the line below. Next, in a random subset of these cases, the percentage of tumour cells staining was scored as 0, 1, 2, 3, 4 (low to high %, as discussed in Materials and methods). The number of tumours at each level of percent cell positivity for c-Myc is indicated on the line below.

densitometry, after Western blot. These data showed c-*myc* gene amplification in 21% of tumours (Jenkins *et al*, 1997), using assays not based on *in situ* discrimination of tumour *vs* nontumour cells. The lower frequency of c-*myc* in this prior study is in contrast with the data we present here, and could be the result of the higher sensitivity and precision of the FISH and immunohistochemical methods, as distinct from quantitative PCR and Western blot densitometry. In addition, the 70% of amplified tumours in our study is also much higher than the 12% reported by Schraml *et al* (1999), using a c-*myc* FISH test on a tissue microarray. This large difference may be because the arrays are prepared from cores of paraffin-embedded tissue, as small as 0.6 mm in diameter which may contain too few tumour cells for complete analysis of amplification of a gene, such as c-*myc*. c-*myc* is known to be quite heterogeneous in its gene amplification within individual tumours (in contrast to *HER2/neu*, for example) (Persons *et al*, 1997).

Most previous reports on the expression of c-*myc* mRNA have utilised Northern blot, dot blot or PCR-based approaches, while just a few involved *in situ* hybridisation, which were primarily performed on frozen tissue sections (Liao and Dickson, 2000). Normal breast tissue is dominated by adipose cells, differing greatly from tumour tissue in its epithelial cellularity. Thus, normal and tumour tissues may not be rigorously compared by techniques involving RNA extraction from total tissue. Therefore, conclusions such as 'increased expression' may be more difficult to make from studies with Northern blot, dot blot and PCR-based techniques that require RNA extraction from tissues that have not been fastidiously micro-dissected for selection of tumour cells. Using a more sensitive, nonradioactive *in situ* hybridisation (ISH) approach on formalin-fixed, paraffin-embedded sections, we report herein high expression of c-*myc* mRNA in 92% of high-grade breast carcinomas. This figure is much higher than the recently reported data (22%), obtained by using a real-time RT–PCR method (Bieche *et al*, 1999). Dilution of the RNA from epithelium by the RNA from adipose in normal breast tissue in this latest prior report may be one of the possible explanations for this large difference.

In conclusion, the present study shows that approximately 70, 92 and 70% of biopsies of untreated high-grade breast cancer exhibit c-*myc* gene amplification, mRNA overexpression and protein overexpression, respectively. In most cases (84%), with gene copy amplification, the c-*myc* gene gains one to two additional copies. c-*myc* gene amplification was significantly associated with expression of its mRNA (both by intensity in invasive cells and by percentage positivity in invasive cells), and with expression of its protein (by percentage positivity in invasive cells). However, our data were also consistent with the prior literature on c-Myc (reviewed in Nass and Dickson, 1997; Liao and Dickson, 2000), indicating complex transcriptional, post transcriptional, translational and post-translational control of c-Myc expression *in vitro*. Specifically, in Table 5 we observed that in 40% of the high-grade tumours tested, c-Myc protein was expressed at high levels, despite a lack of its gene amplification.

It will be interesting to analyse lower grade tumours and premalignant lesions, with the same measurement tools, to determine if this c-*myc* amplification pattern is different, comparing different steps in onset and progression of the disease. Specifically, prior studies in fibroblasts and in human mammary epithelial cells (Liao *et al*, 1998, 2000a, b) have demonstrated that only a subtle deregulation of expression of c-Myc is sufficient to allow genomic instability. These prior cell biologic findings raise the question of whether c-Myc protein expression precedes or follows its gene amplification during the course of the natural history of breast cancer. It will also be interesting for future studies of lower grade breast cancers and premalignant lesions to determine whether there is evidence of nuclear exclusion of c-Myc protein. Indeed, nuclear exclusion of c-Myc in high-grade tumours could serve to attenuate its functions in later stages of disease progression (Liao and Dickson, 2000).

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REFERENCES

Augenlich L, Wadler S, Gorner G, Richards C, Ryan L, Multani A, Pathak S, Benson A, Haller D, Heerdt B (1997) Low-level c-*myc* amplification in human colonic carcinoma cell lines and tumors: a frequent, p53-independent mutation associated with improved outcome in a randomized multi-institutional trial. *Cancer Res* **57**: 1769–1775

Aulmann S, Bentz M, Sinn HP (2002) C-myc oncogene amplification in ductal carcinoma *in situ* of the breast. *Breast Cancer Res Treat* **74**: 25–31

Berns E, Klijn J, Van Putten W, van Staveren I, Portengen H, Foekens J (1992) c-*myc* amplification is a better prognostic factor than *HER2/neu* amplification in primary breast cancer. *Cancer Res* **52**: 1107–1113

Bieche I, Laurendeau I, Tozlu S, Olivi M, Vidaud D, Lidereau R, Vidaud M (1999) Quantitation of MYC gene expression in sporadic breast tumors with a real-time transcription-PCR assay. *Cancer Res* **59**: 2759–2765

Chana J, Cree I, Foss A, Hungerford J, Wilson G (1998a) The prognositc significance of c-*myc* oncogene expression in uveal melanoma. *Melanoma Res* **8**: 139–144

Chana J, Grover R, Wilson G, Hudson D, Forders M, Sanders R, Grobbelaar A (1998b) The clinical significance of c-*myc* oncogene expression in melanomas of the scalp. *Br J Plast Surg* **51**: 191–194

Chana J, Wilson G, Cree I, Alexander R, Myatt N, Neale M, Foss A, Hungerford J (1999) c-*myc*, p53, and *bcl-2* expression and clinical outcome in uveal melanoma. *Br J Opthamol* **83**: 110–114

Deming S, Nass S, Dickson R, Trock B (2000) c-*myc* amplification in breast cancer: a meta-analysis of its occurrence and prognostic relevance. *Br J Cancer* **83**: 1688–1695

Diebold J, Suchy B, Baretton G, Blasenbreu S, Meier W, Schmidt M, Rabes H, Lohrs U (1996) DNA ploidy and MYC DNA amplification in ovarian carcinomas: correlation with p53 and bcl-2 expression, proliferative activity and prognosis. *Virchows Arch* **429**: 221–227

Grover R, Chana J, Grobbelaar A, Hudson D, Forders M, Wilson G, Sanders R (1999) Measurement of c-*myc* oncogene expression provides an accurate prognostic marker for acral lentiginous melanoma. *Br J Plast Surg* **52**: 122–126

Grover R, Ross D, Wilson G, Sanders R (1997) Measurement of c-*myc* oncoprotein provides an independent prognostic marker for regional metastatic melanoma. *Br J Plast Surg* **50**: 478–482

Hyman E, Kauraniemi P, Hautaniemi S, Wolf M, Mousses S, Rozenblum E, Ringner M, Sauter G, Monni O, Elkahloun A, Kallioniemi O, Kallioniemi A (2002) Impact of DNA amplification on gene expression patterns in breast cancer. *Cancer Res* **62**: 6240–6245

Isola J, Chu L, De Vries S, Matsumura K, Chew K, Ljung B, Waldman F (1999) Genetic alterations in ERBB2-amplified breast carcinomas. *Clin Cancer Res* **5**: 4140–4145

Jenkins R, Qian J, Lieber M, Bostwick D (1997) Detection of c-*myc* oncogene amplification and chromosomal anomalies in metastatic

prostatic carcinoma by fluorescence *in situ* hybridization. *Cancer Res* **57**: 524–531
Liao D, Dickson R (2000) c-Myc in breast cancer. *Endocrine-Relat Cancer* **7**: 143–164
Liao D, Hou X, Bai S, Li S, Li J (2000a) Unusual deregulation of cell cycle components in early and frank estrogen-induced renal neoplasias in the Syrian hamster. *Carcinogenesis* **21**: 2167–2173
Liao D, Natarajan G, Deming S, Jamerson M, Johnson M, Chepko G, Dickson R (2000b) Cell cycle basis for the onset and progression of c-Myc-induced, TGFa-enhanced mouse mammary gland carcinogenesis. *Oncogene* **19**: 1307–1317
Liao D, Pantazis C, Hou X, Li S (1998) Promotion of estrogen-induced mammary gland carcinogenesis by androgen in the male Noble rat: probable mediation by steroid receptors. *Carcinogenesis* **19**: 2173–2180
Marcu K, Bossone S, Patel A (1992) Myc function and regulation. *Annu Rev Biochem* **61**: 809–860
Melhem M, Meisler A, Finley G, Bryce W, Jones M, Tribby I, Pipas J, Koski R (1992) Distribution of cells expressing Myc proteins in human colorectal epithelium, polyps, and malignant tumors. *Cancer Res* **52**: 5853–5864
Nass S, Dickson R (1997) Defining a role for c-Myc in breast tumorigenesis. *Breast Cancer Res Treat* **44**: 1–22
Nesbit C, Tersak J, Prochownik E (1999) MYC oncogenes and human neoplastic disease. *Oncogene* **18**: 3004–3016
Persons D, Borelli K, Hsu P (1997) Quantitation of *HER-2/neu* and c-*myc* gene amplification in breast carcinoma using fluorescence *in situ* hybridization. *Mod Pathol* **10**: 720–727
Pietilainen T, Lipponen P, Aaltomaa S, Eskelinen M, Kosma V, Syrjanen K (1995) Expression of c-*myc* proteins in breast cancer as related to established prognostic factors and survival. *Anticancer Res* **15**: 959–964
Polacarz S, Hey N, Stephenson T, Hill A (1989) c-*myc* oncogene product $p62^{c\text{-}myc}$ in ovarian mucinous neoplasms: immunohistochemical study correlated with malignancy. *J Clin Pathol* **42**: 148–152
Pollack JR, Sorlie T, Perou CM, Rees CA, Jeffrey SS, Lonning PE, Tibshirani R, Botstein D, Borresen-Dale AL, Brown PO (2002) Microarray analysis reveals a major direct role of DNA copy number alteration in the transcriptional program of human breast tumors. *Proc Natl Acad Sci USA* **99**: 12963–12968
Sartelet S, Grossi L, Pasquier D, Conbaret V, Bouvier R, Ranchere D, Plantaz D, Munzer M, Philip T, Birembaut P, Zahm J, Bergeron C, Gaillard D, Pasquier B (2002) Detection of N-myc amplification by FISH in immature areas of fixed neuroblastomas: more efficient than Southern blot/PCR. *J Pathol* **198**: 83–91
Sasano H, Nagura H, Silverberg S (1992) Immunolocalization of c-*myc* oncoprotein in mucinous and serous adenocarcinomas of the ovary. *Hum Pathol* **23**: 491–495
Sato H, Tsuchiya A, Abe R (1995) Correlation between c-*myc* protein expression and phases of the cell cycle in human colorectal carcinomas. *Fukushima J Med Sci* **41**: 111–113
Schraml P, Kononen J, Bubendorf L, Moch H, Bissig H, Noctito A, Mihatsch M, Kallioniemi O, Sauter G (1999) Tissue microarrays for gene amplification surveys in many different tumor types. *Clin Cancer Res* **5**: 1966–1975
Sikora K, Chan S, Evan G, Gabra H, Markham N, Stewart J, Watson J (1987) c-*myc* oncogene expression in colorectal cancer. *Cancer* **59**: 1289–1295
Sikora K, Evan G, Stewart J, Watson J (1985) Detection of the c-*myc* oncogene product in testicular cancer. *Br J Cancer* **52**: 171–176
Smith D, Goh H-S (1996) Overexpression of c-*myc* proto-oncogene in colorectal carcinoma is associated with a reduced mortality that is abrogated by point mutation of the p53 tumor suppressor gene. *Clin Cancer Res* **2**: 1049–1053
Sundaresan V, Forgacs I, Wight D, Wilson B, Evan G, Watson J (1987) Abnormal distribution of c-*myc* oncogene product in familial adenomatous polyposis. *J Clin Pathol* **40**: 1274–1281
Visca P, Alo P, Del Nonno F, Bott C, Trombetta G, Marandino F, Filippi S, Di Tondo U, Donnorso R (1999) Immunohistochemical expression of fatty acid synthase, apoptotic-regulating genes, proliferating factors, and *ras*, protein product in colorectal adenomas, carcinomas, and adjacent nonneoplastic mucosa. *Clin Cancer Res* **5**: 4111–4118
Visscher D, Wallis T, Awussah S, Mohamed A, Crissman J (1997) Evaluation of MYC and chromosome 8 copy number in breast carcinoma by interphase cytogenetics. *Genes, Chromosomes, Cancer* **6**: 389–404
Voravud N, Foster C, Gilbertson J, Sikora K, Waxman J (1989) Oncogene expression in cholangiocarcinoma and in normal hepatic development. *Hum Pathol* **20**: 1163–1168
Watson J, Stewart J, Evan G, Ritson A, Sikora K (1986) The clinical significance of low cytometric c-*myc* oncoprotein in testicular cancer. *Br J Cancer* **53**: 331–337
Williams A, Piris J, Wyllie A (1990) Immunohistochemical demonstration of altered intracellular localization of the c-*myc* oncogene product in human colorectal neoplasms. *J Pathol* **160**: 287–293
Yuen M, Wu P, Lau J, Lai C (2001) Expression of c-Myc, c-Fos, and c-Jun in hepatocellular carcinoma. *Cancer* **91**: 106–112
Zar JH (1974) *Biostatistical Analysis*. Prentice-Hall: Englewood Cliffs, NJ

MOLECULAR BIOLOGY OF THE CELL

fourth edition

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Front cover Human Genome: Reprinted by permission from *Nature*, International Human Genome Sequencing Consortium, 409:860–921, 2001 © Macmillan Magazines Ltd. Adapted from an image by Francis Collins, NHGRI; Jim Kent, UCSC; Ewan Birney, EBI; and Darryl Leja, NHGRI; showing a portion of Chromosome 1 from the initial sequencing of the human genome.

Back cover In 1967, the British artist Peter Blake created a design classic. Nearly 35 years later Nigel Orme (illustrator), Richard Denyer (photographer), and the authors have together produced an affectionate tribute to Mr Blake's image. With its gallery of icons and influences, its assembly created almost as much complexity, intrigue and mystery as the original. *Drosophila, Arabidopsis,* Dolly and the assembled company tempt you to dip inside where, as in the original, "a splendid time is guaranteed for all." (Gunter Blobel, courtesy of The Rockefeller University; Marie Curie, Keystone Press Agency Inc; Darwin bust, by permission of the President and Council of the Royal Society; Rosalind Franklin, courtesy of Cold Spring Harbor Laboratory Archives; Dorothy Hodgkin, © The Nobel Foundation, 1964; James Joyce, etching by Peter Blake; Robert Johnson, photo booth self-portrait early 1930s, © 1986 Delta Haze Corporation all rights reserved, used by permission; Albert L. Lehninger, (unidentified photographer) courtesy of The Alan Mason Chesney Medical Archives of The Johns Hopkins Medical Institutions; Linus Pauling, from Ava Helen and Linus Pauling Papers, Special Collections, Oregon State University; Nicholas Poussin, courtesy of ArtToday.com; Barbara McClintock, © David Micklos, 1983; Andrei Sakharov, courtesy of Elena Bonner; Frederick Sanger, © The Nobel Foundation, 1958.)

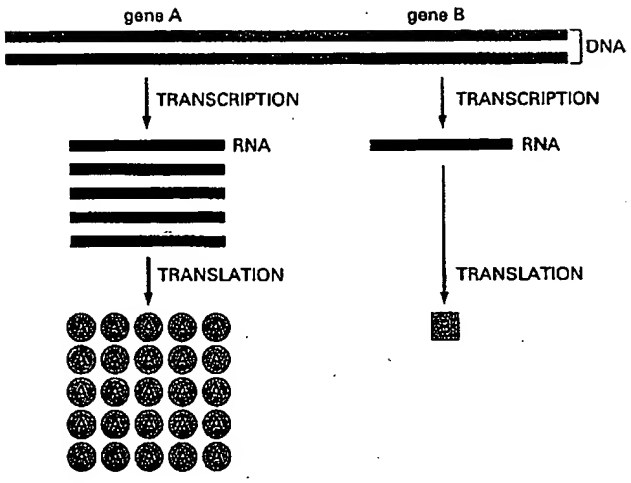

Figure 6–3 Genes can be expressed with different efficiencies. Gene A is transcribed and translated much more efficiently than gene B. This allows the amount of protein A in the cell to be much greater than that of protein B.

FROM DNA TO RNA

Transcription and translation are the means by which cells read out, or express, the genetic instructions in their genes. Because many identical RNA copies can be made from the same gene, and each RNA molecule can direct the synthesis of many identical protein molecules, cells can synthesize a large amount of protein rapidly when necessary. But each gene can also be transcribed and translated with a different efficiency, allowing the cell to make vast quantities of some proteins and tiny quantities of others (Figure 6–3). Moreover, as we see in the next chapter, a cell can change (or regulate) the expression of each of its genes according to the needs of the moment—most obviously by controlling the production of its RNA.

Portions of DNA Sequence Are Transcribed into RNA

The first step a cell takes in reading out a needed part of its genetic instructions is to copy a particular portion of its DNA nucleotide sequence—a gene—into an RNA nucleotide sequence. The information in RNA, although copied into another chemical form, is still written in essentially the same language as it is in DNA—the language of a nucleotide sequence. Hence the name **transcription**.

Like DNA, RNA is a linear polymer made of four different types of nucleotide subunits linked together by phosphodiester bonds (Figure 6–4). It differs from DNA chemically in two respects: (1) the nucleotides in RNA are *ribonucleotides*—that is, they contain the sugar ribose (hence the name *ribo*nucleic acid) rather than deoxyribose; (2) although, like DNA, RNA contains the bases adenine (A), guanine (G), and cytosine (C), it contains the base uracil (U) instead of the thymine (T) in DNA. Since U, like T, can base-pair by hydrogen-bonding with A (Figure 6–5), the complementary base-pairing properties described for DNA in Chapters 4 and 5 apply also to RNA (in RNA, G pairs with C, and A pairs with U). It is not uncommon, however, to find other types of base pairs in RNA: for example, G pairing with U occasionally.

Despite these small chemical differences, DNA and RNA differ quite dramatically in overall structure. Whereas DNA always occurs in cells as a double-stranded helix, RNA is single-stranded. RNA chains therefore fold up into a variety of shapes, just as a polypeptide chain folds up to form the final shape of a protein (Figure 6–6). As we see later in this chapter, the ability to fold into complex three-dimensional shapes allows some RNA molecules to have structural and catalytic functions.

Transcription Produces RNA Complementary to One Strand of DNA

All of the RNA in a cell is made by DNA transcription, a process that has certain similarities to the process of DNA replication discussed in Chapter 5.

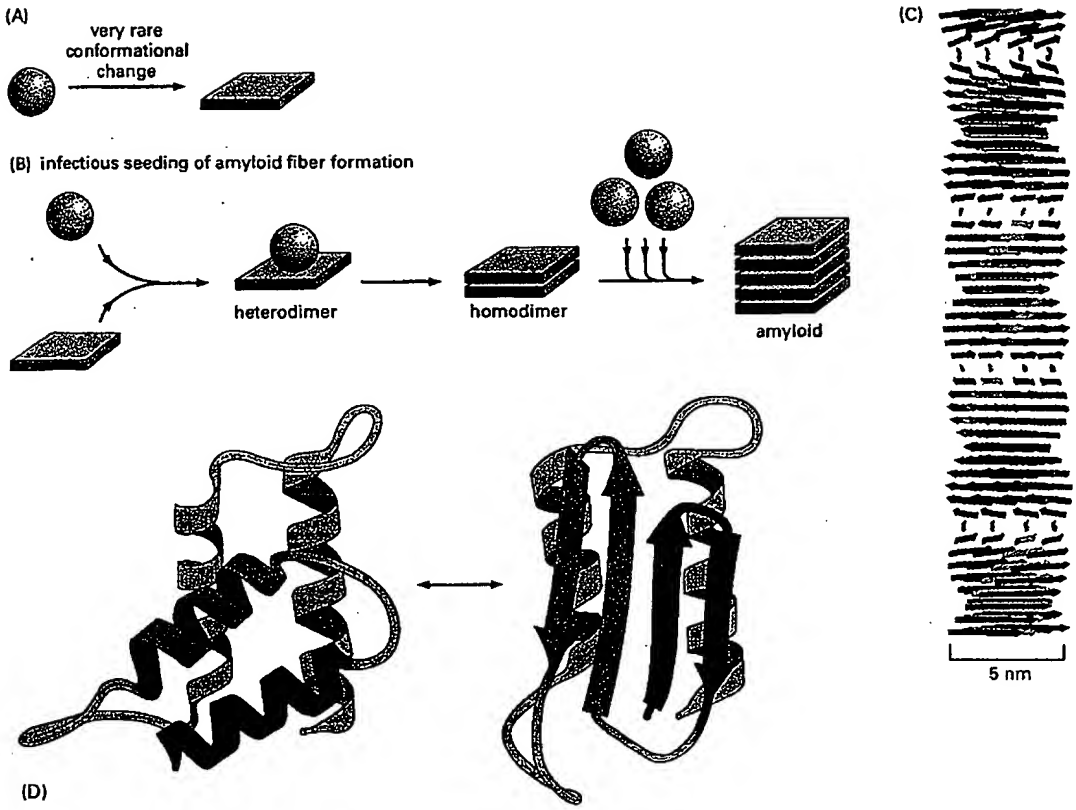

Figure 6–89 Protein aggregates that cause human disease. (A) Schematic illustration of the type of conformational change in a protein that produces material for a cross-beta filament. (B) Diagram illustrating the self-infectious nature of the protein aggregation that is central to prion diseases. PrP is highly unusual because the misfolded version of the protein, called PrP*, induces the normal PrP protein it contacts to change its conformation, as shown. Most of the human diseases caused by protein aggregation are caused by the overproduction of a variant protein that is especially prone to aggregation, but because this structure is not infectious in this way, it cannot spread from one animal to another. (C) Drawing of a cross-beta filament, a common type of protease-resistant protein aggregate found in a variety of human neurological diseases. Because the hydrogen-bond interactions in a β sheet form between polypeptide backbone atoms (see Figure 3–9), a number of different abnormally folded proteins can produce this structure. (D) One of several possible models for the conversion of PrP to PrP*, showing the likely change of two α-helices into four β-strands. Although the structure of the normal protein has been determined accurately, the structure of the infectious form is not yet known with certainty because the aggregation has prevented the use of standard structural techniques. (C, courtesy of Louise Serpell, adapted from M. Sunde et al., *J. Mol. Biol.* 273:729–739, 1997; D, adapted from S.B. Prusiner, *Trends Biochem. Sci.* 21:482–487, 1996.)

animals and humans. It can be dangerous to eat the tissues of animals that contain PrP*, as witnessed most recently by the spread of BSE (commonly referred to as the "mad cow disease") from cattle to humans in Great Britain.

Fortunately, in the absence of PrP*, PrP is extraordinarily difficult to convert to its abnormal form. Although very few proteins have the potential to misfold into an infectious conformation, a similar transformation has been discovered to be the cause of an otherwise mysterious "protein-only inheritance" observed in yeast cells.

There Are Many Steps From DNA to Protein

We have seen so far in this chapter that many different types of chemical reactions are required to produce a properly folded protein from the information contained in a gene (Figure 6–90). The final level of a properly folded protein in a cell therefore depends upon the efficiency with which each of the many steps is performed.

We discuss in Chapter 7 that cells have the ability to change the levels of their proteins according to their needs. In principle, any or all of the steps in Fig-

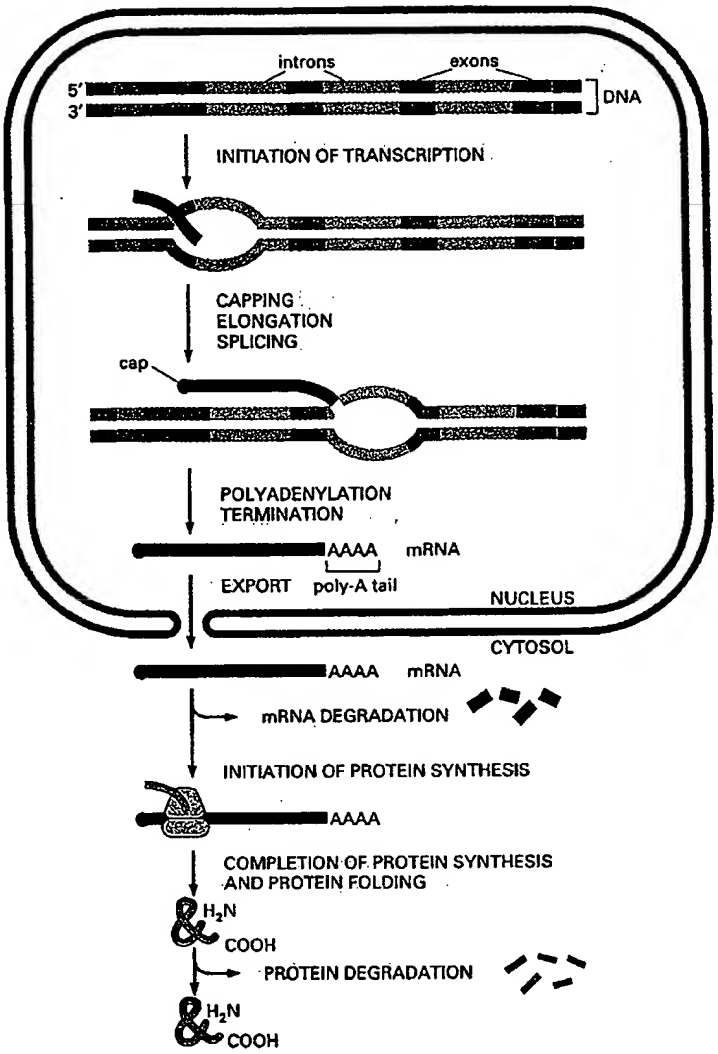

Figure 6–90 The production of a protein by a eucaryotic cell. The final level of each protein in a eucaryotic cell depends upon the efficiency of each step depicted.

ure 6–90) could be regulated by the cell for each individual protein. However, as we shall see in Chapter 7, the initiation of transcription is the most common point for a cell to regulate the expression of each of its genes. This makes sense, inasmuch as the most efficient way to keep a gene from being expressed is to block the very first step—the transcription of its DNA sequence into an RNA molecule.

Summary

The translation of the nucleotide sequence of an mRNA molecule into protein takes place in the cytoplasm on a large ribonucleoprotein assembly called a ribosome. The amino acids used for protein synthesis are first attached to a family of tRNA molecules, each of which recognizes, by complementary base-pair interactions, particular sets of three nucleotides in the mRNA (codons). The sequence of nucleotides in the mRNA is then read from one end to the other in sets of three according to the genetic code.

To initiate translation, a small ribosomal subunit binds to the mRNA molecule at a start codon (AUG) that is recognized by a unique initiator tRNA molecule. A large ribosomal subunit binds to complete the ribosome and begin the elongation phase of protein synthesis. During this phase, aminoacyl tRNAs—each bearing a specific amino acid bind sequentially to the appropriate codon in mRNA by forming complementary base pairs with the tRNA anticodon. Each amino acid is added to the C-terminal end of the growing polypeptide by means of a cycle of three sequential

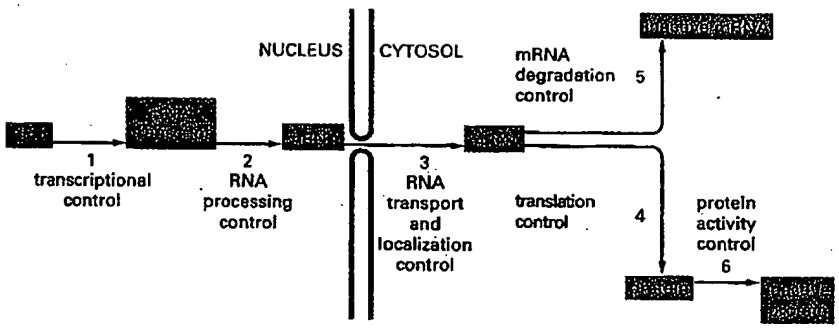

Figure 7–5 Six steps at which eucaryotic gene expression can be controlled. Controls that operate at steps 1 through 5 are discussed in this chapter. Step 6, the regulation of protein activity, includes reversible activation or inactivation by protein phosphorylation (discussed in Chapter 3) as well as irreversible inactivation by proteolytic degradation (discussed in Chapter 6).

Gene Expression Can Be Regulated at Many of the Steps in the Pathway from DNA to RNA to Protein

If differences among the various cell types of an organism depend on the particular genes that the cells express, at what level is the control of gene expression exercised? As we saw in the last chapter, there are many steps in the pathway leading from DNA to protein, and all of them can in principle be regulated. Thus a cell can control the proteins it makes by (1) controlling when and how often a given gene is transcribed (**transcriptional control**), (2) controlling how the RNA transcript is spliced or otherwise processed (**RNA processing control**), (3) selecting which completed mRNAs in the cell nucleus are exported to the cytosol and determining where in the cytosol they are localized (**RNA transport and localization control**), (4) selecting which mRNAs in the cytoplasm are translated by ribosomes (**translational control**), (5) selectively destabilizing certain mRNA molecules in the cytoplasm (**mRNA degradation control**), or (6) selectively activating, inactivating, degrading, or compartmentalizing specific protein molecules after they have been made (**protein activity control**) (Figure 7–5).

For most genes transcriptional controls are paramount. This makes sense because, of all the possible control points illustrated in Figure 7–5, only transcriptional control ensures that the cell will not synthesize superfluous intermediates. In the following sections we discuss the DNA and protein components that perform this function by regulating the initiation of gene transcription. We shall return at the end of the chapter to the additional ways of regulating gene expression.

Summary

The genome of a cell contains in its DNA sequence the information to make many thousands of different protein and RNA molecules. A cell typically expresses only a fraction of its genes, and the different types of cells in multicellular organisms arise because different sets of genes are expressed. Moreover, cells can change the pattern of genes they express in response to changes in their environment, such as signals from other cells. Although all of the steps involved in expressing a gene can in principle be regulated, for most genes the initiation of RNA transcription is the most important point of control.

DNA-BINDING MOTIFS IN GENE REGULATORY PROTEINS

How does a cell determine which of its thousands of genes to transcribe? As mentioned briefly in Chapters 4 and 6, the transcription of each gene is controlled by a regulatory region of DNA relatively near the site where transcription begins. Some regulatory regions are simple and act as switches that are thrown by a single signal. Many others are complex and act as tiny microprocessors, responding to a variety of signals that they interpret and integrate to switch the neighboring gene on or off. Whether complex or simple, these switching devices

occur in the germ line, the cell lineage that gives rise to sperm or eggs. Most of the DNA in vertebrate germ cells is inactive and highly methylated. Over long periods of evolutionary time, the methylated CG sequences in these inactive regions have presumably been lost through spontaneous deamination events that were not properly repaired. However promoters of genes that remain active in the germ cell lineages (including most housekeeping genes) are kept unmethylated, and therefore spontaneous deaminations of Cs that occur within them can be accurately repaired. Such regions are preserved in modern day vertebrate cells as CG islands. In addition, any mutation of a CG sequence in the genome that destroyed the function or regulation of a gene in the adult would be selected against, and some CG islands are simply the result of a higher than normal density of critical CG sequences.

The mammalian genome contains an estimated 20,000 CG islands. Most of the islands mark the 5′ ends of transcription units and thus, presumably, of genes. The presence of CG islands often provides a convenient way of identifying genes in the DNA sequences of vertebrate genomes.

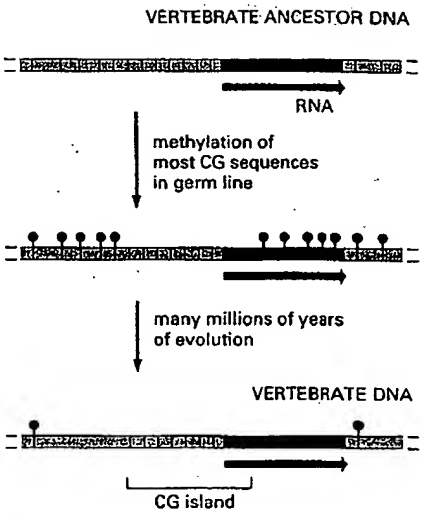

Figure 7–86 A mechanism to explain both the marked overall deficiency of CG sequences and their clustering into CG islands in vertebrate genomes. A *black line* marks the location of a CG dinucleotide in the DNA sequence, while a *red* "lollipop" indicates the presence of a methyl group on the CG dinucleotide. CG sequences that lie in regulatory sequences of genes that are transcribed in germ cells are unmethylated and therefore tend to be retained in evolution. Methylated CG sequences, on the other hand, tend to be lost through deamination of 5-methyl C to T, unless the CG sequence is critical for survival.

Summary

The many types of cells in animals and plants are created largely through mechanisms that cause different genes to be transcribed in different cells. Since many specialized animal cells can maintain their unique character through many cell division cycles and even when grown in culture, the gene regulatory mechanisms involved in creating them must be stable once established and heritable when the cell divides. These features endow the cell with a memory of its developmental history. Bacteria and yeasts provide unusually accessible model systems in which to study gene regulatory mechanisms. One such mechanism involves a competitive interaction between two gene regulatory proteins, each of which inhibits the synthesis of the other; this can create a flip-flop switch that switches a cell between two alternative patterns of gene expression. Direct or indirect positive feedback loops, which enable gene regulatory proteins to perpetuate their own synthesis, provide a general mechanism for cell memory. Negative feedback loops with programmed delays form the basis for cellular clocks.

In eucaryotes the transcription of a gene is generally controlled by combinations of gene regulatory proteins. It is thought that each type of cell in a higher eucaryotic organism contains a specific combination of gene regulatory proteins that ensures the expression of only those genes appropriate to that type of cell. A given gene regulatory protein may be active in a variety of circumstances and typically is involved in the regulation of many genes.

In addition to diffusible gene regulatory proteins, inherited states of chromatin condensation are also used by eucaryotic cells to regulate gene expression. An especially dramatic case is the inactivation of an entire X chromosome in female mammals. In vertebrates DNA methylation also functions in gene regulation, being used mainly as a device to reinforce decisions about gene expression that are made initially by other mechanisms. DNA methylation also underlies the phenomenon of genomic imprinting in mammals, in which the expression of a gene depends on whether it was inherited from the mother or the father.

POSTTRANSCRIPTIONAL CONTROLS

In principle, every step required for the process of gene expression could be controlled. Indeed, one can find examples of each type of regulation, although any one gene is likely to use only a few of them. Controls on the initiation of gene transcription are the predominant form of regulation for most genes. But other controls can act later in the pathway from DNA to protein to modulate the amount of gene product that is made. Although these **posttranscriptional controls**, which operate after RNA polymerase has bound to the gene's promoter and begun RNA synthesis, are less common than *transcriptional control*, for many genes they are crucial.